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Interactions Between Irrigation Fluids and Endodontic Sealers: Assessing Biological, Physicomechanical and Chemical aspects

Thesis submitted for the degree of Philosophiae Doctor

Department of Endodontics
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Preface

This thesis is submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor at the Department of Endodontics, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo. The research presented here was conducted at the University of Oslo, the University of Birmingham (UK), and the Nordic Institute of Dental Materials AS, under the supervision of Dr Pia Titterud Sunde, Dr Håkon Valen, and Dr Josette Camilleri

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The present dissertation is the result of interdisciplinary work undertaken at the *Department of Endodontics, Faculty of Dentistry, University of Oslo*; *Nordic Institute of Dental Materials (NIOM), Oslo*; and *School of Dentistry, University of Birmingham, UK,* during the years 2018-2022. The fascinating journey started in 2016 when after a 6-month research internship at NIOM, professors Dag Ørstavik and Pia Titterud Sunde first presented the potential of me performing a Ph.D. programme at the University of Oslo. The next milestone happened when my upcoming supervisors, Pia Titterud Sunde and Håkon Valen (NIOM), encouraged me to pursue a Doctoral Research Fellow position at the Faculty of Dentistry and take up the challenge of a PhD. I will forever be grateful for the honour of working and collaborating with such wonderful people and highly knowledgeable researchers. You all crafted my passion for research in Endodontology. Your unconditional emotional and academic support was the key to my wellness and continuous improvement (Kaizen). You were always there to put the "derailed" train back on track.

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In Norway, I found an organised and well-equipped environment that fit my work ethic and desire to work hard. Apart from the work aspect, Norway is also a great place in terms of work and life balance. I am fascinated by Norwegian society's openness, politeness and inclusiveness. I am grateful to the Norwegian people for helping me further explore these values and be well-integrated.

My cultural horizons were broadened by meeting new people, exploring their customs and travelling. As Dalai Lama said, “Home is where you feel at home and are treated well”. Norway is my home.

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My family. To my mother, Kiki, my father, Dimitris and my brother Dimitrakis. You make my world go round. I see the world through your eyes. I breathe the oxygen of life through your soul. I am so proud of you.

To science. Research is driven by the innate impulsion of people for hope and thanks to research we people live on the honey of hope. Paraphrasing one of my favourite poems, "the most fascinating scientific milestones are those we haven't yet reached, they are the ones yet to come". I would like to dedicate the original poem to all those who contribute to science. Thank you for everything.

"The most beautiful sea has not yet been crossed.

The most beautiful child hasn't grown up yet.

We haven't seen our best days yet.

And the most beautiful words I wanted to say to you, I haven't said yet.”

Nâzım Hikmet

Vasilis Kapralos
Oslo, October 2023

List of Papers

Paper I

Kapralos V, Valen H, Ørstavik D, Koutroulis A, Camilleri J, and Sunde PT. “Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate”. In: Dental Materials. Vol. 37, (2021). DOI: 10.1016/j.dental.2020.11.011

Paper II

Kapralos V, Valen H, Koutroulis A, Camilleri J, Ørstavik D, and Sunde PT. “The dentine-sealer interface: Modulation of antimicrobial effects by irrigation”. In: International Endodontic Journal. Vol. 55, (2022). DOI: 10.1111/iej.13692

Paper III

Kapralos V, Sunde PT, Camilleri J, Morisbak E, Koutroulis A, Ørstavik D, and Valen H. “Effect of chlorhexidine digluconate on antimicrobial activity, cell viability and physical properties of three endodontic sealers”. In: Dental Materials. Vol. 38, (2022). DOI: 10.1016/j.dental.2022.04.013

Abbreviations

ADT	**agar diffusion test**
BADGE	**bisphenol A diglycidyl ether**
$Ca(H_2PO_4)_2$	**calcium biphosphate**
$Ca(OH)_2$	**calcium hydroxide**
Ca^+	**calcium ion**
$Ca_{10}(PO_4)_6(OH)_2$	**calcium hydroxyapatite**
CaO	**calcium oxide**
CFU	**colony forming unit**
CHX	**chlorhexidine digluconate**
Cl	**chlorine**
CO_2	**carbon dioxide**
COD	**Crystallography Open Database**
CSLM	**confocal scanning laser microscopy**
DCT	**direct contact test**
DMSO	**dimethyl sulfoxide**
DNA	**deoxyribonucleic acid**

E. faecalis	***Enterococcus faecalis***
EDS	**energy dispersive spectroscopy**
EDTA	**ethylenediaminetetraacetic acid**
EPS	**extracellular polymeric substances**
FTIR	**Fourier transform infrared spectroscopy**
gf	**gram-force**
H_2O	**hydrogen dioxide**
HEDP	**1-hydroxyethane 1,1-diphosphonate**
HOCl	**hypoclorous acid**
ISO	**International Organization for Standardization**
MCE	**mixed cellulose esters**
MTA	**mineral trioxide aggregate**
MTAD	**mixture of Doxycycline, citric acid and a detergent**
MTT	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide**
Na^+	**sodium ion**
NaCl	**sodium chloride, saline**

NaOCl	**sodium hypochlorite**
NH^-_2	**amino groups**
OCl^-	**hypochlorite ions**
OD	**optical density**
PBS	**phosphate-buffered saline**
PCS	**Pulp Canal Sealer**
qPCR	**quantitative polymerase chain reaction**
Ra	**arithmetic roughness**
S. aureus	***Staphylococcus aureus***
S. epidermidis	***Staphylococcus epidermidis***
S. mutans	***Streptococcus mutans***
SEM	**scanning electron microscopy**
SH	**sulfhydryl groups**
Si	**silicon**
SiO_2	**silicon dioxide**
SR1	**Sulfur-River 1**
TSB	**tryptone soy broth**

VHN	**Vickers hardness number**
W	**tungsten**
XRD	**X-ray diffraction**
Zn	**zinc**
ZOE	**zinc oxide eugenol**
Zr	**zirconium**

Summary of papers

Paper I focuses on the antimicrobial and physicochemical characterisation of the tested endodontic sealers after chlorhexidine digluconate (CHX) exposure. Remnants of irrigation solutions are present in the root canal system after the completion of chemo-mechanical root canal preparation. CHX in 2% concentration is often used in endodontics as a final irrigant before the placement of endodontic sealers. Due to binding to dentin and subsequent release of CHX, this may influence the sealers' properties. The primary aim of this *in vitro* study was to assess the antibacterial, physical (physicomechanical), surface and chemical properties of AH Plus, BioRoot RCS and Pulp Canal Sealer (PCS) after exposure to 2% CHX. An *ex vivo* tooth model and endo training blocks were used to simulate irrigation procedures.

Contact with CHX increased the antibacterial activity of all sealers investigated and affected their physicomechanical properties. PCS alone and in contact with CHX exerted the highest antibacterial activity against both planktonic bacteria and biofilms. BioRoot RCS was the sealer that was affected to various extents in physicomechanical and surface properties compared to the other two sealers investigated. Surface characterisation showed that both AH Plus and BioRoot RCS remained unchanged under CHX irrigation, whilst two additional phases were observed for PCS.

The potential interaction between CHX and endodontic sealers with various chemistries underlines the need to customise clinical protocols regarding irrigation techniques and materials used. The individualisation of root canal treatments will ensure that root canal fillings maintain their antimicrobial properties over time without compromising their physicochemical performance.

Paper II demonstrates how the antimicrobial effects at the dentine-sealer interface are modulated by irrigation. Most *in vitro/ex vivo* study designs in the literature have investigated instrumentation, irrigation and obturation as separate entities. In the clinical situation, they are strongly related to each other. Clinically, different irrigation protocols are often combined with various obturation materials. Dentine as well as many sealers have antibacterial properties. The irrigants used may affect the chemistry of dentine and sealer surfaces and compromise or enhance their antimicrobial properties. This study aimed to use an *ex vivo* tooth model to assess whether the residual presence of 1% NaOCl or 2% CHX may augment or reduce the antibacterial properties of dentine and three endodontic sealers. A second aim was to compare whether/how residuals from two irrigation protocols, namely, "1% NaOCl followed by 17% EDTA (1% NaOCl + 17% EDTA)" and "1% NaOCl followed by 17% EDTA and 2% CHX (1% NaOCl + 17% EDTA + 2% CHX)", could alter the antibacterial effect of dentine or sealers.

The split tooth model developed for this study was found to be reproducible. NaOCl and CHX affected to various extents the antimicrobial properties of dentine as well as sealer surfaces, and the two irrigation protocols differed in antimicrobial efficacy. Overall, CHX improved the antibacterial activity of sealer and dentine surfaces.

Although many *in vitro* and *ex vivo* studies have demonstrated a wide range of antibacterial efficacy among endodontic materials, clinical studies indicate no significant differences regarding the clinical outcome among endodontic sealers and irrigation solutions. The success of endodontic treatment is multifactorial, with each distinct procedural step playing a significant role and contributing to the overall therapeutic result. Clinical studies need to address the potential clinical advantages of antimicrobial endodontic sealers.

Paper III evaluates the effect of CHX on antimicrobial activity, cell viability and physicochemical properties of the tested endodontic sealers. Constituents from irrigation liquids may interact with sealers and affect their physicochemical and biological properties. Moreover, 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, along the dentine-sealer interface or periapical tissues. This study aimed to assess the antibacterial activity and cytotoxicity (cell viability) of the leachates of the three sealers with and without CHX contact and investigate the effect of CHX on sealers' water uptake, sorption, solubility, porosity, surface characteristics and pH of the immersion liquid.

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. 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, which was decreased.

Sealer leachates should be investigated further, including thorough chemical characterization 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. Further studies involving more complex environments, such as tooth models and the use of human cells or clinical bacterial isolates, may give an insight into the role of sealer leachates in the therapeutics of endodontic pathosis.

Contents

Chapter 1

Introduction

The oral cavity is a continuation of the skin/mucosa to the external environment and is perceived as the first barrier for keeping irritants away from the interior parts of the human body and maintaining homeostasis. The teeth constitute an exceptional/multifunctional organ of the oral cavity, the mutual part of the digestive and respiratory systems. They serve distinct integral functions of the human body, such as mastication, articulation of specific sounds and contribute as well to the aesthetics of the face.

A remarkable aspect of tooth anatomy is the coexistence of outer hard tissues (enamel, dentine, cementum) with the vital soft tissues in the core, the pulp. The dental hard tissues that constitute the calcified structure of the teeth serve as a protective barrier for the pulp and maintain the integrity and vitality of the soft tissue. Dentin and pulp are developmentally highly interdependent and constitute the mature pulp-dentin complex, which plays an essential role in preserving a tooth viable and functional in the oral cavity [1]. The periapical area (periapex), i.e., the periodontal tissues and bone surrounding the apex of the root, is closely associated with the dental pulp and crucial for the maintenance of teeth.

1.1 Endodontic microbiota

Apical periodontitis is a chronic inflammatory disease of periradicular tissues caused by microorganisms [2]. The most common cause of pulp injury is microbial and notably from bacteria deriving from carious lesions (Figure 1.1) [3, 4]. Endodontic infection is defined as the microbial infection of the root canal system of a tooth. The engagement of microbes in the initiation, development and persistence of apical periodontitis has been extensively described in the scientific literature. Bacteria can enter the root canal space through the coronal (most common), lateral or apical routes as well as dentinal tubules [5]. The influx of microorganisms into the root canal space as a sequel of caries, trauma, and periodontal diseases or operative procedures can cause pulpal inflammation and lead to reversible or irreversible pulpitis [6]. Due to a lack of active circulation, a necrotic pulp loses its ability to mobilize inflammation and defend against the colonisation of oral bacteria [7]. Therefore, an endodontic infection is established,

and bacteria travelling through apical and lateral foramina contact the periradicular tissues initiating an inflammatory response in the area, which leads to the formation of diverse types of apical periodontitis [8].

Antonie van Leeuwenhoek [1632–1723], the inventor of single-lens microscopes, was the first to observe oral microbiota [9]. He was a pioneer in oral microbiology when 400 years ago, he shaped the first insights into the microbes of dental plaques. He had characteristically described: “The crown of this tooth was nearly all decayed, while its roots consisted of two branches so that the very roots were uncommonly hollow and the holes in them were stuffed with a soft matter. I took this stuff out of the hollows in the roots and mixed it with clean rainwater, and set it before the magnifying glass to see if there were as many living creatures in it as I had afore time discovered, and I must confess that the whole stuff seemed to me to be alive” [10]. Back then, despite Leeuwenhoek’s findings, the role of microbes in the aetiopathology of apical periodontitis was unknown.

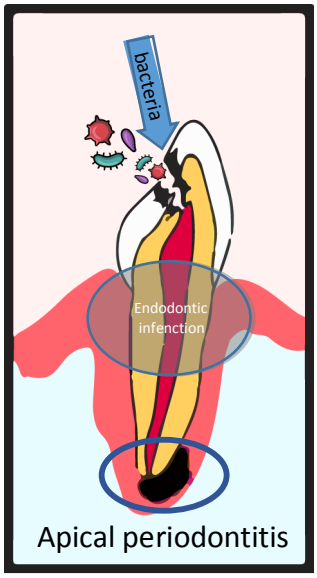

Figure 1.1: Illustration of apical periodontitis development. The most common cause of pulp injury is microbial and notably from bacteria in carious lesions.

Only after approximately 200 years, Willoughby Dayton Miller, an American dentist, published his classic study about the relationship of bacteria with apical periodontitis [11]. He observed, utilising bacterioscopy of the root canals, the presence of the three basic bacterial morphotypes, i.e., cocci, bacilli and spirilla. He wrote: “We assume, in a general way, that bacteria must in some manner be connected with these processes (pulp diseases). There are, then, as I have already pointed out, different species of bacteria in the diseased pulp that have not yet been cultivated on artificial media and of whose pathogenesis we know nothing definite. Their great numbers in some pulps, and especially the repeated occurrence of spirochaetes, justify the supposition that, under certain circumstances, they may play an important role in suppurative processes.” With his findings, Miller was the first to raise the hypothesis that bacteria were the causative factors of apical periodontitis and that the bacterial microflora was clearly divergent at the various levels (coronal, middle, apical) of the

roots. The causal relationship between bacteria and apical periodontitis was indicated in the classic study in germ-free rats by Kakehashi *et al.* [12]. A further step to establishing bacteria as the cause of apical periodontitis was performed by Sundqvist who used anaerobic cultivation methods to assess the microbiology of intact human teeth that were devitalised after trauma [13]. Another study that provided strong evidence about the relationship between bacteria and apical periodontitis was performed by Möller *et al.* in 1981 [4]. Using monkey's teeth as a model, the authors showed that only devitalised pulps that were infected led to apical periodontitis. In contrast, uninfected pulps did not cause any pathological changes in the periapex.

Since then, oral microbiology has been through many advances, from the first time *Streptococcus mutans* was isolated from a healthy oral cavity in 1924 [14] to systematic efforts with the use of modern molecular techniques for the complete decoding of oral bacteria. In the last years, international institutes [(U.S. National Institute of Health and International Human Microbiota Consortium] have combined efforts to identify and register the human microbiome in databases. These efforts have led to the development of organ-specific microbial databases, including the Human Oral Microbiome Database [http://www.homd.org] [15]. The oral cavity is an environment where more than 700 bacterial species have been identified and constitute the human oral microbiome [16]. The aetiopathogenesis of endodontic diseases is polymicrobial, as bacteria, viruses [17-20], fungi and more recently, archaea have been found in endodontic infections [21, 22]. However, bacteria constitute most of the microorganisms implicated in the aetiology of endodontic diseases. Theoretically, all the bacteria hosted in the oral cavity can invade the pulp, infect the root canal system and cause apical periodontitis. The endodontic microflora differs from individual to individual, suggesting that multiple bacterial profiles can lead to disease development. Data sets from culture and molecular studies indicate the polybacterial character of endodontic infections, with approximately 500 unique bacterial taxa, from 100 genera and nine phyla identified [23]. *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* are the predominant phyla. The anaerobic bacteria are dominant in the root canal flora of necrotic pulps. However, molecular analyses indicate that endodontic infections are opportunistic rather than specific as a range of species participate [24, 25]. The vast majority of microbes that are isolated from infected vital pulps are streptococci and staphylococci [26]. Still, many others, including anaerobes, can be identified nowadays by using new molecular techniques [27].

Endodontic infections can be classified as intraradicular and extraradicular depending on the location of the infection related to the root canal. Apical periodontitis can be classified as either primary or post-treatment disease based on whether it is associated with untreated or treated root canals. Both primary and post-treatment diseases have a bacterial aetiopathology and this has been documented by numerous microscopic studies [28-30], culture [7, 31, 32] and molecular techniques [33-35]. Notably, for post-treatment apical periodontitis, the exact molecular studies have shown that most root canal infections can be located within the root canal system (persistent or secondary intraradicular) and, in few cases, to the periradicular tissues (extraradicular).

Multispecies bacterial communities usually occur in both primary and persistent/secondary endodontic infections. Nevertheless, studies have shown that primary infections harbour a higher diversity in microbiota than persistent/secondary [36-41]. Apical periodontitis has a heterogeneous aetiology as increased variability in the composition of endodontic bacterial communities has been shown from individual to individual [37-39, 42, 43]. Culture and molecular methods have verified the association of many cultivable bacterial species with apical periodontitis and included new candidate pathogens such as fastidious cultivate species and as-yet uncultivated bacteria [23].

1.1.2 Primary apical periodontitis

Primary intraradicular infection will, if untreated, lead to primary apical periodontitis. Approximately 500 microorganisms have been identified in endodontic infections, most of them bacteria. In primary intraradicular infections, 10 to 30 species have been detected per canal using culture based and molecular techniques [44], and more recent pyrosequencing studies show even higher diversity reaching 100 species or even more per canal [33, 45, 46]. Symptomatic apical periodontitis has been shown to harbour a higher number of microbial species than asymptomatic cases, and the bacterial load varies from 10^3 to 10^8 per canal in the asymptomatic cases [47, 48] while in symptomatic ranges between 10^4 to 10^9 [49-51]. The bacterial numbers and diversity are proportionally associated with the size of the periapical lesion [52, 53].

Bacterial species isolated from primary infections fall into 9 of the 13 phyla that have oral representatives, namely *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Spirochaetes* *Fusobacteria*, *Synergistetes*, *Proteobacteria*, *SR1* and "*Candidatus Saccharibacteria*" (formerly TM7) [47, 52, 54-58]. However, pyrosequencing studies have revealed representatives of at least nine other phyla in endodontic infections [33, 39, 45, 59, 60]. Nevertheless, species derived from uncommon phyla are not high-abundance members of the endodontic community. The most abundant bacterial taxa in primary infections comprise black-pigmented gram-negative anaerobic species (*Prevotella* and *Porphyromonas* species such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis*), other gram negative species (*Fusobacterium nucleatum*, *Dialister* species, *Tannerella forsythia* and *Treponema* species) and gram-positive bacteria (*Parvimonas micra*, *Filifactor alocis*, *Pseudoramibacter alactolyticus*, *Olsenella uli*, *Actinomyces* species, *Streptococcus* species, *Propionibacterium* species and *Cutibacterium acnes*) [6, 21, 46, 47, 52, 54-58, 61-72]. Additionally, culture-independent molecular studies have revealed several as-yet-uncultivated or uncharacterized bacteria [44]. These bacteria comprise approximately 55% of the taxa found in infected root canals of teeth with primary apical periodontitis and represent about 38% of the community members [54]. Interestingly, among others, Bacteroidaceae [G-1], bacterium HMT 272 (or Bacteroidetes clone X083) as well as members of the Synergistetes and Spirochaetes phyla, are the most prevalent bacteria in primary infections [52, 61, 73].

1.1.3 Post-treatment disease

Persistent or secondary intraradicular infections or even extraradicular infections can lead to post-treatment apical periodontitis [74]. Most, if not all, cases with apical periodontitis lesions harbour an intraradicular infection [31-34, 75-78]. Moreover, if microorganisms have not been sufficiently eradicated after instrumentation and are present at the filling time, there is a high probability of adverse treatment outcomes [79-82].

Various bacterial species can lead to post-treatment apical periodontitis, since high interindividual variability in biofilm communities has also been reported in treated teeth [36, 37]. However, compared to primary infections, studies have demonstrated less variability in bacterial communities, notably in cases with adequate endodontic treatments. To the contrary, inadequately treated teeth present bacterial diversity similar to teeth with primary apical periodontitis [31, 32, 36, 37]. Bacterial load in treated teeth with previous apical periodontitis may vary from 10^3 to 10^7 per canal, while higher numbers are observed in inadequately treated cases. Gram-positive bacteria are the most prevalent in samples isolated before the root canal filling procedure [40, 83-85]. These pathogens can potentially affect the outcome of root canal treatment.

Enterococcus faecalis

E. faecalis is frequent in teeth with post-treatment apical periodontitis (90% of the cases) [33, 34, 78, 85-91], and *E. faecalis* is nine times more likely to be present in root canals associated with post-treatment than in primary disease [90]. This finding may be partially explained by the fact that *E. faecalis* is a robust microorganism and can grow in various environments [92, 93].

However, recent scientific data question the role of *E. faecalis* as the most important pathogen in treatment failure and post-treatment apical periodontitis [20, 94]. Some studies have not detected any enterococci in endodontically treated teeth associated with apical periodontitis. Other community-profiling studies have shown that *E. faecalis* is not dominant in most re-treatment cases [34, 36, 37, 95]. Moreover, quantitative real-time PCR (qPCR) studies demonstrate high variability as this species may make for no more than 1 % to 100% of the total microbiota [25, 34, 85, 96].

Bacterial phyla in post-treatment disease

Post-treatment apical periodontitis has also been associated with other bacterial phyla that can be abundant in post-treatment infections: streptococci which in many cases can be the dominant species [32, 75, 76, 84, 97], *Arachnia propionica* (Propionibacterium pionicum),

species, *Actinomyces* species, *Cutibacterium acnes*, *Pseudoramibacter alactolyticus*, *Fusobacterium nucleatum, Parvimonas micra, Pseudoramibacteralactolyticus, Filifactor alocis Dialister* and *Prevotella* species [31, 32, 34, 36, 41, 57, 76, 86]. As yet-uncultivated or uncharacterized phylotypes correspond to 55% of the taxa encountered in treated canals and may account for the dominant species in some cases [36].

It has been shown that in acute forms of the disease, when an abscess is formed, the intraradicular infection has exited the root canal system and expanded extraradicularly in the periradicular tissues [98]. An acute apical abscess is a characteristic example of an extraradicular infection. However, there is controversy in endodontic literature whether an extraradicular infection may occur in asymptomatic (chronic) forms of the disease [99]. In post-treatment apical periodontitis, studies have found extraradicular formation of biofilms upon the exterior apical surfaces [100-102] with present persistent symptomatology or signs (sinus tract) [103].

Extraradicularly, many anaerobic bacteria that are also commonly found in intraradicular infections have been reported with the use of culture [104-107] and molecular techniques [108-112]. Histobacteriologic studies have demonstrated that extraradicular biofilms are present in approximately 6 % of teeth associated with apical periodontitis [28, 100]. In untreated cases or teeth under treatment, extraradicular infections are also associated with persistent symptoms [28, 100, 113]. In most cases, extraradicular biofilms may be “supplied” with bacteria by an intraradicular biofilm as they morphologically appear to be similar to bacteria in the apical part of the roots [28, 114]. In a few cases reported in the literature, there was no association between extraradicular biofilms and bacteria derived from intraradicular infections [113, 115]. These infections led to persistent post-treatment disease and were treated surgically.

1.2 Biofilm in endodontic disease

The bacteria that ingress into the root canal system are primarily organised in biofilms (Figure 1.2). A biofilm is a sessile multicellular microbial community characterized by cells firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substances (EPS) [116]. Most of the microbial species in nature live in metabolically integrated biofilm communities; the human body is no exception [117]. Bacteria form biofilms as a defence, protection from the host immune system and increased resistance to antimicrobial agents [118]. Planktonic bacteria appear to be related to acute infections in the human body, whilst biofilms are associated with chronic infections, controlled inflammation, and limited tissue damage [119-121]. Biofilms are responsible for 65-80% of human infectious diseases such as prostatitis, osteomyelitis, and orthopaedic device-associated infections [119, 122, 123]. Caries, gingivitis, and marginal periodontitis are typical examples of biofilm-induced diseases in the oral cavity, with apical periodontitis being recently included in the set of suchlike diseases [28, 124]. Bacterial cells in biofilms are aggregated in microcolonies distributed throughout the EPS matrix according to the availability of nutrients, metabolic interactions among the community members, and arrival time. The vast majority of cells are located close to the surface where

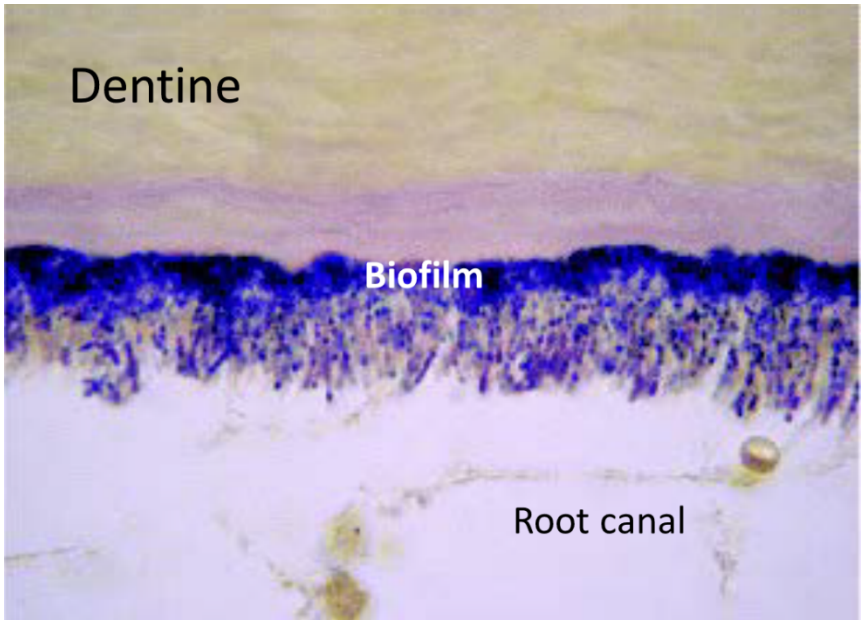

Figure 1.2: The bacteria that ingress into the root canal system are primarily getting organised in biofilms. Ricucci D, Siqueira JFJ. Endodontology: An integrated biological and clinical view. Germany: Quintessence Publishing Co. Ltd; 2013

the biofilm has adhered. The biofilm structure is traversed by water channels that carry water and nutrients, and drain the waste products. The bulk of a biofilm is mainly composed of the EPS matrix (>90%), and the cells constitute <10% of the biofilm mass [125]. Polysaccharides are the main constituents of the EPS matrix, with proteins, nucleic acids, and lipids also present. The EPS matrix plays a vital role within the biofilm community by serving multiple functions. It enhances surface adhesion, bolsters mechanical stability and structural organisation, facilitates the accumulation of extracellular enzymes involved in nutrient acquisition and defense against antimicrobial agents, fosters positive intercellular interactions by facilitating close communication between cells, acts as a nutrient reservoir, retains moisture, acts as a protective barrier against antimicrobial substances and host defenses, and supports the expansion of the biofilm [125-127]. As for resistance to host defences, bacteria in biofilms present enhanced resistance against phagocytosis by neutrophils [128] and macrophages [129]. Biofilm cells exhibit altered gene expression compared to their planktonic counterparts [130-132]. This difference in gene expression leads to a different phenotype characterised by low growth rate for the biofilm and increased resistance to antimicrobials, host defence and environmental stress [133]. Bacteria in biofilms can coordinate their functions by active communication between them (quorum sensing) or with the host using chemical signalling molecules called autoinducers [134]. This interaction system builds up the functions and physiology of the biofilms and their virulence. Both gram-positive and gram-negative bacterial species participate in this communication system [134-136].

The concept of biofilm formation in the aetiopathogenesis of apical periodontitis has gained popularity throughout the years and has attracted the attention of research efforts [137-143]. Nair was the first to identify structures that resembled biofilms in infected root

canals of teeth with apical periodontitis [143], and the apparent association of apical periodontitis in primary and post-treatment disease with the presence of bacteria in biofilms in the apical third of the canal was demonstrated by Ricucci and Siqueira in 2010 [28]. Bacterial biofilms are prevalent in the apical portion of canals in infected teeth which have not been treated and teeth with apical periodontitis. However, it remains unclear whether the development of apical periodontitis necessitates the presence of bacteria in communities. Apical periodontitis meets most criteria that classify it as a disease caused by biofilms [28, 144, 145]. But there are some cases of apical periodontitis where biofilm communities cannot be detected. This knowledge questions whether biofilms are needed for apical periodontitis to develop. Based on current literature and taking into account that biofilms are difficult to eliminate, we can conclude that there is a strong association between apical periodontitis and biofilms [120, 146, 147].

Intraradicular biofilms are composed of several layers. Morphologically, the biofilms differ from individual to individual as different bacterial species produce different amounts of EPS, affecting the thickness of the matrix [125, 148, 149]. Biofilms in root canal system are polymicrobial. The diversity of pathogens has been associated with different clinical manifestations of apical periodontitis, e.g. the communities related to the asymptomatic disease are significantly different in variety from those occurring in symptomatic cases [42, 45, 54]. Moreover, post-treatment disease presents other bacterial community profiles from those in primary apical periodontitis [36, 37].

The extension of an intraradicular infection and the subsequent bacterial colonization of the external root surface give rise to extraradicular biofilms (extraradicular infections). Intraradicular biofilms are present in most root canals of both untreated and treated teeth with apical periodontitis, while extraradicular biofilms are not prevalent as often [28]

Different bacteria's combined action initiates apical periodontitis in a multispecies biofilm [121]. The community's species and the interactions between them modulate the virulence in the biofilm matrix [150]. When bacteria colonise the periapex and get organised in biofilms, virulence factors from their biomass diffuse and may induce or prolong the inflammation in periapical tissues [151].

The anatomical complexity of the root end prevents the host from easily accessing the anatomic site of infection, a fact that makes for the persistent nature of biofilm infections. The vicinity of bacterial biofilms to the periradicular tissues gives rise to inflammatory responses.

Bacterial communities are engaged in synergistic interactions among their members to protect against host defence antimicrobial agents. Moreover, bacterial communities related to inflammatory responses, known as inflammophilic, have even been suggested to take advantage of inflammation which may favour their survival and persistence [152].

1.3 Endodontic treatment

Endodontics (from the Greek roots endo- "inside" and odont- "tooth") is the discipline in dentistry concerned with pulp-periapical biology and pathology as well as the prevention and treatment of endodontic infections. The ultimate goal of endodontic therapy is to heal apical periodontitis: remove pulpal remnants and bacteria by mechanical debridement/chemical irrigation and create a bacteria- and fluid-tight seal in the root canal space [153]. Endodontic pathosis is primarily treated by nonsurgical endodontic treatment (root canal therapy) and retreatment in case of failure [154].

The procedure of endodontic treatment comprises mechanical cleaning (instrumentation), irrigation with root canal irrigants and obturation with filling materials (gutta-percha and endodontic sealers) [155, 156]. Root canal treatment reduces the bacterial load of the infected root canal, which subsequently reduces inflammation of periapical tissues and promotes periapical healing. Mechanical instrumentation removes residual bacteria, pulp tissue and debris, and shapes the root canal walls to facilitate adequate irrigation and obturation [157]. However, mechanical debridement leaves untouched areas [158] and numerous irrigation regimens are used to aid the mechanical debridement in removing bacteria and necrotic pulp tissue [159]. Technically, endodontic treatment aims to establish a hermetic seal from the coronal to the apical end of the treated tooth to prevent bacteria from ingressing into the root canal system. Even vital pulp therapy procedures can be considered preventive measures for root canal infection and development of apical periodontitis. Prevention of apical periodontitis is the aim when the pulp tissues need to be partially or entirely removed in cases of vital teeth. Treatment of apical periodontitis is the aim in cases of established apical disease. Historically, the histopathological classification of apical periodontitis played an important role in determining the prognosis of non-surgical root canal treatment. This perception has driven the classification of apical periodontitis into three categories: apical granuloma, apical cyst, and apical abscess. Clinical diagnosis of apical periodontitis based on symptoms and signs is more realistic to determine the treatment plan and the prognosis as the histopathological diagnosis is not feasible in everyday clinical practice.

Endodontic treatment failure of primary endodontic infection can lead to either a secondary or persistent infection. Breaches in the chain of asepsis during first time treatment and/or recontamination are the principal causes of secondary disease [2]. When endodontic treatment fails, endodontic surgery may be applied. Surgical endodontics includes apicectomy and retrograde filling [154]. Apicectomy involves the removal of the apical part of a root with anatomical complications and/or undebrided canal walls if the root cannot be entirely sealed through either an orthograde or non-surgical method [160, 161].

1.4 Irrigation in Endodontics

Irrigation aims to reach and impact the areas left untouched by mechanical instrumentation and remove debris and smear layer from dentinal walls before sealers can be placed. Various irrigation solutions such as sodium hypochlorite (NaOCl), chlorhexidine digluconate (CHX), 17% ethylene diamine tetracetic acid (EDTA), citric acid and MTAD (mixture of Doxycycline, citric acid and a detergent) are used in endodontic treatments [159, 162], prior to root canal filling. After completion of chemo-mechanical root canal preparation, remnants of irrigation solutions are present in the root canal system [163, 164]. In a recently published review [165], Boutsioukis and Moliz summarized the requirements for the ideal irrigant:

• Strong antimicrobial action against a broad spectrum of microorganisms, both planktonic and those organized in biofilms

• Inactivation of bacterial virulence factors, such as endotoxins and lipoteichoic acids

• Disruption or removal of the biofilm

• Dissolution of pulp tissue remnants

• Removal of accumulated hard-tissue debris and the smear layer or prevention of their formation

• Lack of adverse effects, both local (on dentine and the periapical tissues) and systemic (toxicity, allergic reactions)

• Wide availability at a low cost

In the next chapters focus will be placed on the irrigation solutions that are relevant to the studies of this thesis, namely NaOCl and CHX.

1.4.1 Sodium Hypoclorite-NaOCl

NaOCl is the most known and used root canal irrigant, and among clinicians it is considered the irrigation solution of choice [166]. NaOCl is a water-based disinfectant compound and has been used as a detergent, surface disinfectant, bleach and deodorizer [167]. It can also serve as an effective lubricant for the instrumentation of the root canals [168]. In aqueous solution, NaOCl ionizes into sodium (Na^+) and hypochlorite ions (OCl^-) which is in equilibrium with hypochlorous acid (HOCl) [169, 170]. Normally, unbuffered NaOCl solutions have an alkaline pH (close to 11-12); thus, hypochlorite is the dominant substance [171]. The high alkalinity of NaOCl causes loss of cell membrane integrity, irreversible enzymatic inhibition, and phospholipid degradation in the lipidic hyperoxidation. These processes are part of events such as saponification, degradation of lipids and fatty acids that result in soap and glycerol, neutralization of amino acids, and chloramination [172]. The HOCl moiety is more active than OCl^- and makes for the antibacterial efficacy of hypochlorites. The available chlorine is dependent on the pH of the solution. [173]

NaOCl is the basic irrigation solution widely used in endodontics as it dissolves organic matter of pulp tissue remnants and has antimicrobial properties at acidic and neutral pH, when most of the chlorine exists as hypochlorous acid (HOCl) [159, 174-178]. Its antimicrobial activity spectrum is broad, including fungi, viruses, protozoa and bacteria [167]. As for oral bacteria, it kills them in a short time when in direct contact [179, 180]. Regarding its antimicrobial mechanism, oxidation of sulfhydryl groups (SH) of the essential bacterial amino acid (cysteine) occurs together with the reaction of chlorine with amino groups (NH^-_2), disrupting bacterial metabolism. Moreover, bacterial DNA is disarranged, being chlorinated by NaOCl [181]. It also reduces bacterial virulence factors such as endotoxins and lipoteichoic acids [182]. However, NaOCl does not exhibit residual antimicrobial activity [183]. While it has both antimicrobial and tissue dissolving properties, it lacks substantive antimicrobial activity [183, 184]. There is controversy regarding the ideal concentration of NaOCl solution. It is used clinically in concentrations ranging from 0.5 % to 8.25% [159, 185-190] and clinicians' preferences are diverse among different countries [166, 191-193]. Laboratory studies conclude that the antimicrobial activity of NaOCl is a matter of its concentration; the higher the concentration the more desirable antimicrobial effects [190, 194, 195]. However, clinical studies have shown that the antimicrobial activity of NaOCl solution is independent of concentration, since lower (0.5%) or higher % (5.25) have similar effectiveness [196, 197]. On the other hand, the higher the concentration, the more undesirable/cytotoxic effects have been reported [198, 199]. NaOCl has been reported to react with the collagen of dentine with an even more pronounced effect after the application of a chelating agent that may alter the physicomechanical properties of dentine [200]. Moreover, NaOCl is a caustic substance, and may cause severe pain and distress if accidentally misplaced into periapical and surrounding tissues [201, 202]. Thus, NaOCl in a concentration of 1-2% combines both good antimicrobial properties and low cytotoxicity. Moreover, *in vitro* studies have demonstrated that 1% NaOCl can dissolve the pulp tissue during root canal treatment [203, 204]. Taking into account the low volume of NaOCl that remains in the root canal system between rinses (estimated to ≤30 μL) and the depletion of free available chlorine in reactions with bacterial communities, dentine surface, pulp tissue remnants and other irrigants [205-207], frequent delivery of fresh irrigant during root canal treatment is desirable [208-210]. Preheating of NaOCl has been suggested especially for low concentrations of the solution, as *in vitro* and *ex vivo* studies have shown promising results regarding its antimicrobial effectiveness [190, 211, 212]. On the other hand, in a clinical setting, the temperature of the solution drops in a short time after application, questioning the long-term clinical value of preheating technique [213, 214].

1.4.2 Chlorhexidine

Chlorhexidine digluconate (CHX) has been used as an alternative to NaOCl in cases where direct contact of irrigants with periapical tissues can occur, and as an adjunct root canal irrigant due to its broad-spectrum antibacterial properties and good biocompatibility [215, 216]. It has the ability to be absorbed and bind to dentine, a property called substantivity, which may contribute to a prolonged antimicrobial effect [159, 217-219].

In particular, CHX possesses broad antimicrobial properties and is often used in endodontics as a final irrigation solution [220, 221]. It binds to dentine, releases gradually [217], and thus may interact with the sealer and modify its properties [222].

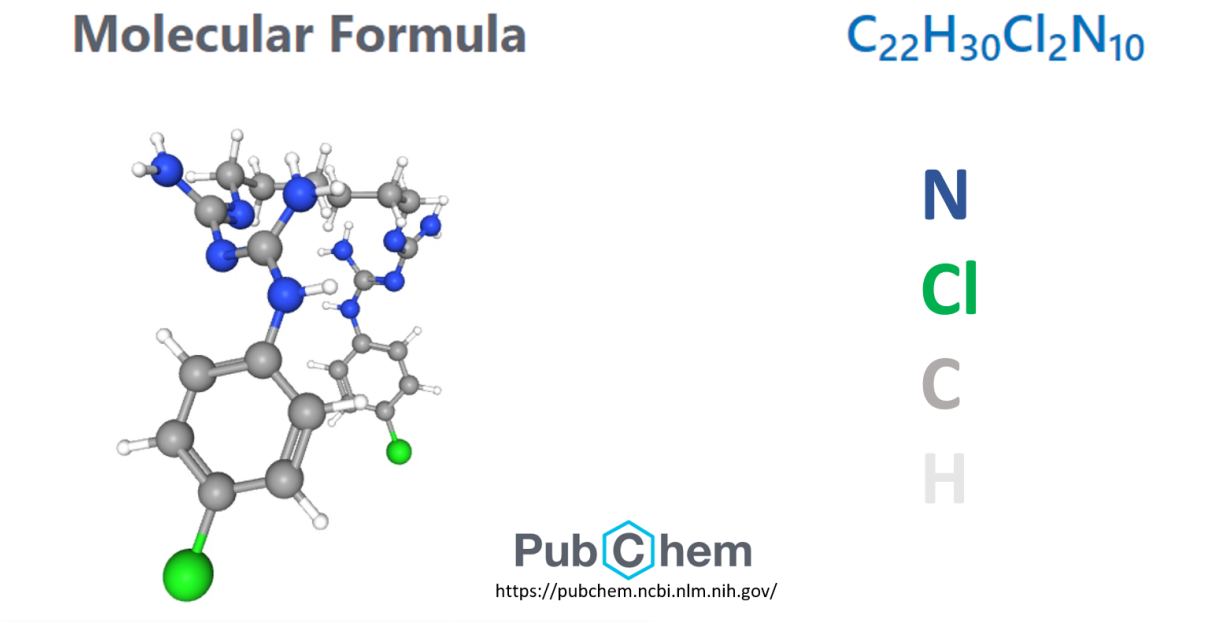

Figure 1.3: Molecular formula of Chlorhexidine. Chlorhexidine is a bisbiguanide compound with a structure consisting of two (p-chlorophenyl) guanide units linked by a hexamethylene bridge. It has a role as an anti-infective agent and an antibacterial agent. It is a member of biguanides and monochlorobenzenes. Retrieved from https://pubchem.ncbi.nlm.nih.gov

CHX 2% is commonly used in clinical practice as final irrigant before filling with endodontic sealers [186, 214]. CHX is a cationic bisbiguanide substance (Figure 1.3) with broad antimicrobial properties as it acts against gram-positive bacteria, gram-negative bacteria, and fungi. Depending on its concentration, it has both bacteriostatic and bactericidal effects [217, 218]. CHX can bind to hard dental tissues and confer lasting antimicrobial properties (up to 12 weeks) to dentine [217-219]. Due to binding to dentin and subsequent release of CHX, this may further modify the sealers' properties. Some *in vitro* studies have concluded that CHX is a stronger antibacterial agent than NaOCl against bacteria [180, 223, 224]. In contrast, more recent studies using multispecies biofilms models showed better results for NaOCl and CHX incapability to disrupt the EPS matrix. [225-227]. This inconsistency in the results [228] may be attributed to methodological limitations such as lack of statistical power, use of different instrumentation and irrigation protocols, or poor sampling procedures [229].

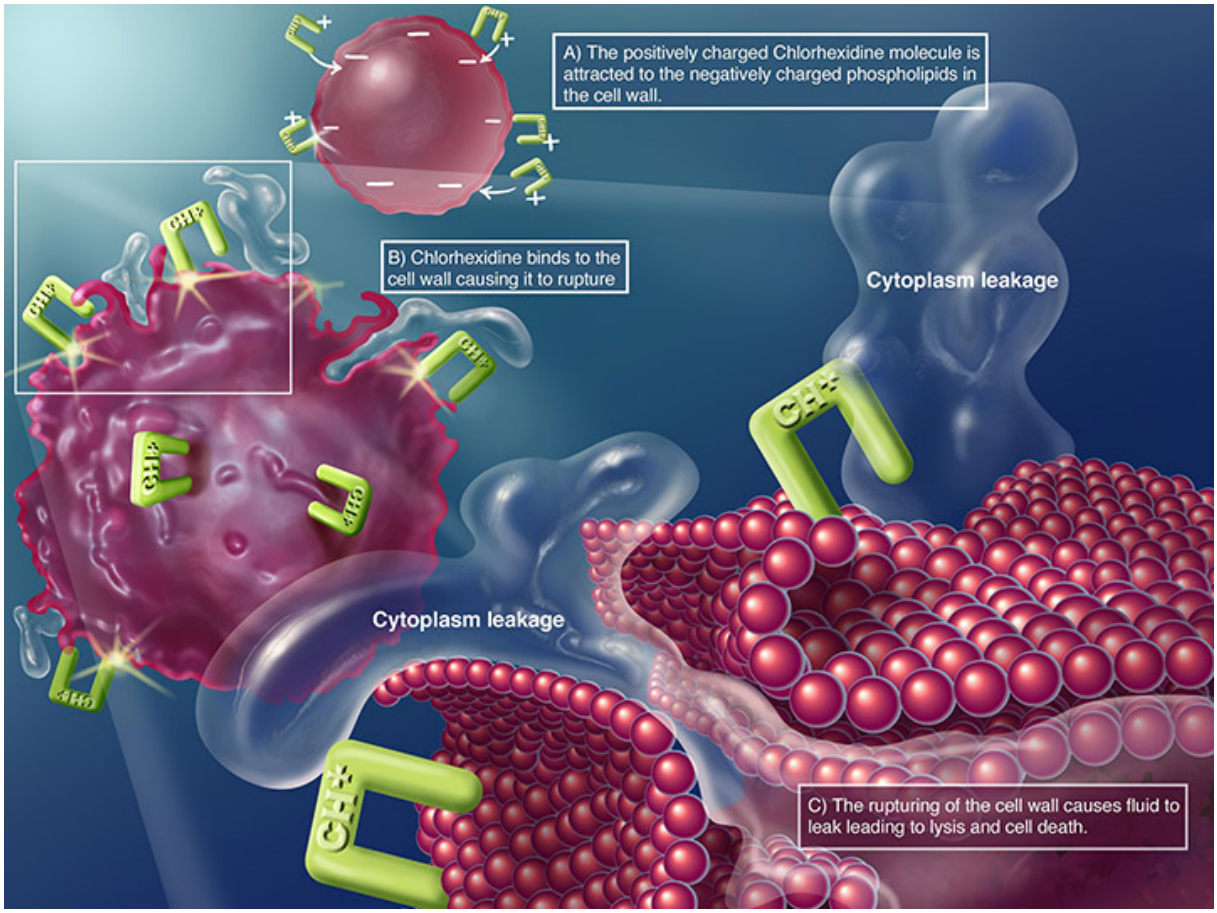

Figure 1.4: Chlorhexidine is positively charged and reacts with the negatively charged microbial cell surface, thereby destroying the integrity of the cell membrane. Subsequently, chlorhexidine penetrates the cell and causes leakage of intracellular components leading to cell death. Since gram-positive bacteria are more negatively charged, they are more sensitive to this agent. Retrieved from: https://chlorhexidinefacts.com/mechanism-of-action.html

CHX is a cationic substance that kills the microbes by acting at the microbial cell wall or outer membrane (Figure 1.4) without being toxic to periapical tissues, unlike the NaOCl [162, 181]. CHX acts against gram-positive bacteria, gram-negative bacteria, and fungi, and has both bacteriostatic and bactericidal effects depending on its concentration [217, 218]. CHX and NaOCl, when in contact, react and lead to the formation of a toxic orange-brown precipitate that may be carcinogenic and cause discolouration [230-232]. Thus, the consecutive use of these two chemicals should be accompanied by intermediate flushes with NaCl or water between each irrigant to achieve thorough disinfection of the root canal system and simultaneously avoid the formation of the toxic precipitate [228].

1.4.3 EDTA

Although NaOCl is the main irrigant of choice for the chemomechanical preparation of the

root canal system, it can dissolve neither dentine debris produced throughout the mechanical preparation of the canal nor the inorganic part of smear layer. Hence, a demineralising agent (chelator) is suggested before root canal filling [165]. The most common demineralising agent used in endodontics is ethylenediamine tetraacetic acid (EDTA) [233, 234]. In concentrations from 15 to 17%, the solution is a potent chelator dissolving both hard-tissue debris and the smear layer [235-237] as well as possesses weak antimicrobial properties [238, 239]. An interesting property of EDTA is its potential anti-biofilm effect since it has been shown to disrupt the biofilm matrix and thus facilitate its detachment [225, 240]. As for biocompatibility, EDTA has been shown to be less cytotoxic than NaOCl [241]. Albeit these favourable properties, there is no evidence to support the use of EDTA or other chelators as the main irrigant. However, some clinicians do use EDTA as the primary irrigation solution during chemomechanical preparation [234]. Regarding interactions between EDTA and NaOCl, it is reported that the levels of free chlorine are dropping rapidly; thus, the alternate use of these two solutions is not recommended also because of deteriorating physical properties of dentine [207, 214, 242].

1.4.4 Irrigation protocols in the clinic

The ideal irrigation protocol should:

- make the irrigant available to the entire root canal system
- hold a constant, frequent refreshment rate of the irrigant to compensate for its depletion
- induce shear stress on the targets (biofilms, debris, smear layer) to detach them from the dentine walls
- facilitate transportation of detached materials of the targets out of the root canal system
- prevent iatrogenic errors that may cause the irrigant extrusion through the apical foramen to the periapical tissues [165].

Worldwide, numerous irrigation protocols have been adopted and used by clinicians. However, NaOCl remains the “gold standard” solution for irrigation use as the primary irrigant of choice. It serves multiple purposes: eliminate microorganisms, disrupt the biofilm structure, dissolve and flush out pulp tissue remnants, remove necrotic tissue, remove the organic components of the smear layer and lubricate the instruments [243, 244]. An essential factor to consider is that the root canal system should always be supplied with fresh NaOCl to keep high levels of free chlorine, which is rapidly depleted due to reactions with organic matter. Thus, copious amounts of NaOCl should be supplied/delivered in the root canal system with the use of a fine needle close to the working length [165]. As aforementioned, the alternate use of NaOCl and chelators, such as EDTA, may lead to the rapid depletion of free chlorine [242, 245]. However, there is still a need for chelation after instrumentation to remove the organic and inorganic parts of the smear layer and disrupt the biofilm matrix. In addition, smear layer removal and exposure of dentinal tubules enhances the adhesion of resin based sealers on dentin walls. Hence, the root canal system should be further rinsed with EDTA

before the canal filling particularly when applying resin- based sealers [246, 247]. Nevertheless, the application of EDTA during the last irrigation phase can disturb the calcium silicate materials' hydration process, leading to a decrease in its hardness, as EDTA has the capacity to chelate calcium [248]. Moreover it has been shown that the use of EDTA is important for the intratubular penetration of AH Plus (epoxy resin based sealer) and thus the antimicrobial properties of the sealer, while BioRoot RCS (calcium silicate based sealer) exhibited antimicrobial properties against intratubular bacteria even in the presence of smear layer [247].

Regarding the last step of chemical preparation and the use of a final rinse after EDTA, there is controversy in the literature. The primary argument against using NaOCl as a final irrigant after ETDA is that it may attack the exposed collagen and lead to dentine erosion [214]. Nevertheless, current scientific data do not support any fracture risk but changes in dentine's superficial layers (changes in the elasticity, strength, or microhardness) [165]. Thus, the clinical significance of such erosion remains unclear, and clinicians currently use protocols both with and without final NaOCl rinse. If deemed necessary, activation of NaOCl by ultrasonic means, or even manually with a guttapercha cone, has been proposed in order to increase the flow of the irrigant in the main canal, to deliver the irrigant farther into remote areas of the root canal system and improve the mechanical cleaning by increasing the wall shear stress [165, 249]. To avoid the potential adverse effects of the alternate use of NaOCl and EDTA, mixtures of NaOCl and weak chelators (MTAD or HEDP) have been suggested. Still, the clinical advantages of such combinations remain unclear [245].

CHX has been considered an alternative to NaOCl for main irrigation, but it has been primarily suggested as a final rinse of the root canal system mainly because it binds to dentine and confers long-lasting antimicrobial properties [81, 217]. Due to potential interactions by the consecutive use of NaOCl, EDTA and CHX [230, 242], extra care is needed by the clinicians to dry the canals before the appliance of the next irrigant or to wash out the canals with the use of NaCl or water in order to avoid any toxic interactions [228].

Even though many *in vitro* studies have shown pronounced antibacterial properties of CHX [180, 223, 250-252], *in vivo* studies have found no statistically significant differences between NaOCl and CHX [25, 253]. CHX has residual substantive antimicrobial effect for up to 12 weeks, while NaOCl confer no long- term antimicrobial effect [183, 184, 217-219]. Moreover, a recent review highlights the use of CHX as the last irrigant in terms of the long-term efficiency of irrigation regimes [254]. Other studies have shown that irrigation with CHX may influence the nature of adherence, adhesion force and "discourage" the subsequent biofilm formation of bacteria upon dentin [252, 255].

The nature of the cases to be treated (primary or post-treatment infection) and the need for broader antimicrobial properties may be key factors for selecting an irrigation regime. *In vitro* studies have shown that NaOCl and CHX are effective against different pathogens [224, 256, 257]. The clinical significance of this is important because in root-filled teeth with infection (post-treatment infection), some persistent bacteria, especially gram-positive facultative bacteria (*Streptococcus* and *Enterococcus* species), are highly prevalent [7,

25, 41, 258]. Thus, the use of irrigants such as CHX that has continued antimicrobial action over time might be of clinical relevance. However, two clinical studies on the retreatment of single-/multirooted teeth with persistent infections showed similar antimicrobial effectiveness and clinical success for NaOCl and CHX [41, 82].

1.5 Endodontic sealers

Traditionally, the most popular technique to seal the canals is to combine two materials, gutta-percha and root canal sealers (endodontic sealers), in which the sealer is responsible for filling the gaps between the gutta-percha and the root canal walls [259]. Newer root-filling techniques, such as the use of single gutta-percha cones are more dependent on sealer properties since the root filling has a large proportion of sealer [247]. Many root canal sealers with various chemistries have been developed and used in endodontics in the pursuit of materials with ideal properties [260]. Grossman back to 1978 published a list of properties of the ideal root canal filling material [261]:

- It should be easily introduced into the canal
- It should seal the canal laterally as well as apically
- It should not shrink after being inserted
- It should be impervious to moisture
- It should be bacteriostatic or at least not encourage bacterial growth [262, 263]
- It should be radiopaque
- It should not stain tooth structure
- It should not irritate periapical tissue [264]
- It should be sterile or quickly and easily sterilised before insertion
- It should be easily removed from the root canal if necessary

Sundqvist and Figdor highlighted three main functions regarding root filling: sealing against ingrowth of bacteria from the oral cavity; entombment of remaining microorganisms; and complete obturation at a microscopic level to prevent stagnant fluid from accumulating and serving as nutrients for bacteria from any source [265].

Physical and chemical properties of endodontic sealers should remain consistent [266-269] with securing the three-dimensional hermetic filling/sealing of the root canals [153, 270]. High solubility to the oral environment of a root canal sealer may lead to a lack of integrity of the material, which in turn may compromise the technical quality of the root filling [266]. This loss of structure creates gaps in the material bulk and along the sealer/dentin or sealer/gutta-percha interface [268], which may create a pathway for microbes and their toxic products into periapical tissues and jeopardise the healing process [266]. In addition, a soluble sealer may be subject to degradation that may further impair its chemical stability [271]; leaching of chemicals may irritate the periapical tissues and increase cytotoxic effects [272].

Irrigation solutions and root canal obturation materials are important for long-lasting clinical success of endodontic treatment [269]. Endodontic sealers based on different

chemical compositions, such as zinc oxide eugenol, resin, silicone or calcium silicate, are available [260].

In the next chapters focus will be on the endodontic sealers that are relevant to the studies of this thesis, namely AH Plus, BioRoot RCS and Pulp Canal Sealer. They represent commonly used materials with different basic composition.

1.5.1 Epoxy resin-based endodontic sealers/AH Plus

Two types of resin-based sealers, epoxy resin-based and methacrylate resin-based, have been used in endodontics. Epoxy resin was invented in Switzerland by chemist P. Castan who was working in “de Trey” (Zurich, Switzerland), leading to the development of the sealer AH 26 by the same company in the 1940s. The first clinical trials of the product were performed in the early 1950s [273]. It is a bis-phenol resin using methenamine (also known as urotropin) for polymerisation, which leaches formaldehyde during setting [274]. The toxic effects (tissue reaction) of the product were first reported in animal studies [275, 276]. This unfavourable release of formaldehyde led to the production of AH Plus, which consists of a mixture of amines that causes polymerisation without forming formaldehyde [153]. AH Plus is the root canal sealer tested the most in the literature and is frequently used as a benchmark for comparisons. It comprises low molecular weight epoxy resins and amines and set by an addition reaction between epoxide groups attached to epoxy resins and amines to form a polymer [277].

The AH sealers series is by far the most successful resin-based sealers [153]. AH Plus is an epoxy-amine resin-based sealer available as a two-component paste either in a paste-paste mixture or an automatic mixing syringe (AH Plus Jet). AH plus paste A contains BADGE (biphenol A diglycidyle ether), calcium tungstate, zirconium oxide, silica, and iron oxide pigments. AH Plus paste B contains amines, silica, and silicone oil. AH Plus possesses excellent physicomechanical properties [278, 279] and sealing ability as it bonds to the dentine (micro-retention). It is a hydrophobic sealer which sets in approximately 8 hours [280], and it has low solubility within the ISO limit. AH Plus exhibits antimicrobial activity during its setting time [281, 282] and various degrees of cytotoxicity when unset [283, 284]. Efforts have been made to enhance the antimicrobial properties of the sealer by incorporating antimicrobial agents such as quaternary ammonium, silver nanoparticles, benzalkonium chloride and CHX [285-288].

1.5.2 Calcium silicate-based sealers/BioRoot RCS

Hydraulic materials are used in endodontics due to their hydration characteristics, namely the formation of calcium hydroxide when mixing with water, and their hydraulic properties. The term hydraulic derives from the greek word “hydra”, which means water; only materials whose primary reaction is with water can be classified as hydraulic.

Different nomenclature has been used for these materials. Mineral trioxide aggregate (MTA) was the first hydraulic material to be marketed, and most refer to hydraulic cements as MTA or MTA-like. Bioceramics are also used, but this term is vague, has been primarily used by manufacturers for advertising their products, and does not describe the chemistry or clinical behaviour of the materials. Moreover, many different dental materials are called bioceramics [289, 290]. What distinguishes hydraulic cements from other materials is their bioactivity, the ability to form hydroxyapatite and have an osteogenic effect [291]. Hence, the term hydraulic, which is also used in the construction industry, is the best way to refer to these types of material [292].

Among the different hydraulic materials, in dentistry and almost exclusively in endodontics, hydraulic calcium silicates (based chiefly on tricalcium silicate) are used. These materials are presented in various consistencies and delivery methods. They are composed primarily of tricalcium and dicalcium silicate and include a radiopacifier, additives, and an aqueous or non-aqueous vehicle. During hydration, tri- and dicalcium silicate react with water to form calcium silicate hydrate and calcium hydroxide [293, 294]. The calcium silicate hydrate is responsible for forming the cement matrix, whilst the calcium hydroxide is leached out and interacts with the environment in which the material is placed [295, 296]:

- $2\ (3\ CaO \cdot SiO_2) + 6\ H_2O \rightarrow 3\ CaO \cdot 2SiO_2 \cdot 3\ H_2O + 3\ Ca(OH)_2$
- $2\ (2\ CaO \cdot SiO_2) + 4\ H_2O \rightarrow 3\ CaO \cdot 2SiO_2 \cdot 3\ H_2O + Ca(OH)_2$

Phosphate in tissues may react with calcium hydroxide and precipitation of calcium phosphate follows the hydration reaction:

- $7\ Ca(OH)_2 + 3\ Ca(H_2PO_4)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12\ H_2O$.

The interaction of hydraulic cements with the clinical environment renders this material unique. Their favourable biological properties (antimicrobial activity and low cytotoxicity) is predominantly related to high alkalinity: calcium hydroxide principally formed out of the hydration process, releases calcium ions (Ca^{+2}) and hydroxyl ions (OH^-) in water for even one month after setting [297]. The ion release can induce hydroxyapatite formation upon the materials' surface, especially when body fluids are present [277]. Regarding radiopacity, bismuth oxide, zirconia, tantalum oxide and barium zirconate have been used, among others [298, 299].

A recent review article [296] classifies the hydraulic cements in endodontics based on the clinical context and the specific environment in which they are used:

- Intracoronal—pulp protection, barrier for regenerative endodontic procedures;

- Intraradicular—root canal sealing, apical plugs;

- Extraradicular—root-end fillers, perforation repair materials.

Hydraulic calcium silicate-based endodontic sealers have been relatively recently introduced in endodontics. The first endodontic sealer of this new class introduced in 2007

was iRoot SP (Innovative Bioceramix) which was associated with the attribute “bioceramic” [295]. Since then, hydraulic sealers with different compositions and delivery systems (pre-mixed or single–paste calcium silicate sealers) have been developed [277]. Despite their high cost, pre-mixed single-paste tricalcium silicate-based sealers are gaining popularity in the clinic as they offer easy handling and time-efficiency compared to the powder liquid hydraulic cements. TotalFill BC (FKG Dentaire), iRoot SP (Innovative Bioceramix), EndoSequence BC (Brasseler) and Edge Endo Sealer (Edge Endo) are the same sealer from the same manufacturer (Innovative Bioceramix), marketed under different brand names. Single-paste calcium silicate-based sealer use a non-aqueous vehicle and sets by interacting with available moisture from dentin tubules that initiates hydration [300]. As long as the material needs to absorb the environmental moisture from the root canal system and then commence the hydration reaction, the release of calcium hydroxide may be delayed [292]. Several single-paste sealers containing tricalcium silicate and organic liquids are marketed: CeraSeal (Meta Biomed), Endoseal MTA (Maruchi), and Bio-C Sealer (Angelus). On the other hand, three powder liquid systems are known: NeoMTA Plus, BioRoot RCS, and Endo CPM [277].

BioRoot RCS is a characteristic powder-liquid representative of the new era of calcium silicate-based sealers, combining good antimicrobial properties and high biocompatibility [271]. Even though some calcium silicate-based sealers have been reported to exceed one month to set completely, BioRoot RCS’ setting time has been measured to approximately 3 hours as it contains calcium chloride that serves as an accelerator of the setting process [301-304]. Hydraulic calcium silicate-based sealers have been reported to present low physicomechanical performance and especially high solubility values. This may be explained by the formation of calcium hydroxide, which has been found to dissolve in the ISO 6876 solubility test [305]. The bioactivity of hydraulic sealers based on calcium silicates results from the presence of soluble components of these materials even after setting, but high solubility of the sealer may further compromise its sealing ability against the ingress of bacteria and reinfection [295]. Although there are conflicting results in the literature, overall the solubility of hydraulic sealers has been reported to be higher than resin-based sealers [306-310]. As for BioRoot RCS, the calcium hydroxide formation and its hydraulic nature render the sealer more susceptible to environmental conditions [311]. Earlier studies have shown high water sorption and porosity for BioRoot RCS, presenting voids in its mass which may compromise the sealer's sealing ability [304, 312]. Nevertheless, the sealing ability of hydraulic calcium silicate sealers, including BioRoot RCS has been reported as comparable to epoxy resin-based sealers [312]. In order to tailor the antimicrobial and physicomechanical properties of hydraulic calcium silicate sealers, nanoparticles or antimicrobial substances (chlorhexidine-hexametaphosphate, chitosan, silver) have been suggested, and modifications of the sealers have been developed [270, 313-315]. No efforts to modify BioRoot RCS with CHX have been reported yet.

1.5.3 Zinc oxide eugenol sealers/Pulp Canal Sealer

Rickert and Dixon developed a zinc oxide-eugenol sealer formula in 1931 which was marketed as Kerr Pulp Canal Sealer [316, 317]. Grossman in 1936 developed his sealer formula that became known under the commercial names Proco-Sol sealer and Roth sealer [318]. To achieve radiopacity, Rickert used silver powder whereas Grossman used bismuth and barium salts [153].

The ZOE materials have been used in dentistry for many years and have been a standard in endodontics, given their long-term success [277]. ZOE sealers comprise zinc oxide powder and eugenol in liquid form, an essential oil derived from cloves [319]. In the moist root canal, the zinc oxide and eugenol react to form an amorphous gel [320] which entraps residual zinc oxide powder, leading to a rigid material structure [321]. In an effort to avoid tooth staining/discoloration caused by silver in powder components, new silver-free formulas were developed: the Grossman formulas, Proco-Sol sealer, Wach's Paste and Tubli-Seal sealer (Kerr, Orange, CA, USA) [277].

Even after 80-90 years since the fabrication of the first ZOE formula, the ZOE sealers remain popular due to their favourable properties, namely antimicrobial properties, fast setting, low cost, and ease of use because of the adequate working time [321]. Even though Roth sealer was discontinued in 2018 [277], many different ZOE sealers are still commercially available, e.g., Pulp Canal Sealer (Kerr), Proco-Sol sealer, Tubli-Seal sealer, Nishika Canal Sealer Eugenol (Nippon Shika Yakuhin, Shimonoseki, Japan), Master-Dent Root Canal (Dentonics, Charlotte, NC, USA) and Pulpdent Root Canal Sealer (Pulpdent, Watertown, MA, USA). Over the years, modifications in ZOE sealers have been introduced to tailor their properties and enhance their clinical performance, with adverse results in many cases [322-327]. Back in 1984, Nambu prepared a prototype ZOE-based root canal sealer with the addition of 1% CHX, achieving higher antimicrobial performance than unmodified ZOE cements [328]. Paraformaldehyde in low concentrations, shown to stimulate pulp cells of residual vital pulp for secondary dentin formation, was added to the N2 sealer and Endométhasone [153, 329]. However, residual paraformaldehyde release can lead to toxic effects in the periapical tissues as it causes coagulative necrosis and affects the reparative potential of the affected areas [329, 330]. Moreover, the controversial N2 sealer, introduced by the Swiss dentist Sargenti [331], had lead and mercury in its composition, and these toxic metals were shown to migrate from periapical tissues to distant organ systems [332]. Thus, N2 was disallowed by the U.S. Food and Drug Administration [333], and the Septodont company developed Endométhasone N without containing paraformaldehyde.

PCS is a traditional eugenol-containing zinc-oxide sealer that has been in clinical use for decades possessing good antimicrobial properties but controversial biocompatibility [322]. Free-leaking eugenol is the first contributing factor to the pronounced antimicrobial efficacy of PCS [334, 335], which has been associated with the high cytotoxicity of ZOE sealers [336]. PCS sets in approximately 2 hours and, as a hydrophobic material, does not favour water adsorption and consequently exhibits low porosity [304].

1.6 Interactions between chlorhexidine and endodontic sealers

Innumer irrigation regimes have been proposed in endodontics, and a multitude of sealers with various chemistries are currently used. Inside the root canals, irrigants and sealers co-exist in tight contact yielding possible interactions [337].

Many studies have tested the effect of irrigation on sealers' properties, such as sealing ability, microleakage, and wettability [5, 262, 338-340]. The irrigants inside the root canal may also alter the sealer chemistry and compromise or enhance their antimicrobial properties. To date, one study tested the effect of water, ethylene diamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) as final irrigants on the antimicrobial activity of three root canal sealers [337]. Release of CHX over time makes it possible that interaction may occur with the sealers used in root canal obturation. There is scant scientific data about the possible impact of CHX on endodontic sealers in relation to antimicrobial efficacy and physicomechanical and chemical properties.

In clinical setting, CHX is mainly used as an auxiliary irrigation solution after NaOCl and EDTA. The interactions between the solutions mentioned above and the dentine have been soundly described in the literature [231, 341]. Since these solutions are applied in succession in endodontic space, interactions can readily occur. Irrigation remnants may be present in the root canal system, as dental practitioners are sometimes unable to adequately dry the canals after irrigation, notably the apical extent of the root canal or anatomical irregularities [163, 164]. From a clinical standpoint, this can also lead to prolonged contact between the last irrigants and the incoming sealers during root canal treatment.

Most of the *in vitro/ex vivo* study designs in the literature investigate instrumentation [342, 343], irrigation [344, 345] and obturation [346, 347] as separate entities when they, in fact, are strongly related to each other [247, 262, 348]. Clinically, different irrigation protocols are often combined with various obturation materials [159, 349, 350]. Both dentine and many sealers have antibacterial properties [282, 337, 351]. The irrigants inside the root canal may affect the chemistry of dentine and sealer surfaces and compromise or enhance their antimicrobial properties [337]. Irrigation liquids may be left in the root canal system (dentinal walls and tubules) after drying, notably in the apical part or anatomical irregularities [163, 164]. In addition, compounds from irrigation liquids are observed on dentin after irrigation [348]. Irrigants and constituents from irrigation liquids may potentially interact with sealers and affect their physiochemical and biological properties. Few studies have investigated the effect of irrigation liquids on antimicrobial properties of sealers and dentin [247, 337, 352] and cytotoxicity of sealers [353, 354]. To date, the combined effect of root canal irrigation and root filling has not been investigated in depth [247].

Chapter 2

Aim of the project

General aim of the study

The overall objective of this thesis was to assess the effects of interactions between three endodontic sealers and irrigation, especially 2% chlorhexidine dicuglonate (CHX), both *in vitro* and *ex vivo*. The primary focus is on the microbiological aspects of these interactions. The secondary aim was to explore further the impact of CHX on cytotoxicity, physicomechanical, and chemical properties of the tested materials.

General hypothesis

The overall null hypothesis was that CHX would cause non-significant changes in any of the antimicrobial, cytotoxic, physical, and chemical properties of tested endodontic sealers in both *in vitro* and *ex vivo* comparative studies performed.

Specific aims

1. Determine the effects of short- and long-term exposure of CHX to sealers' surfaces *in vitro* on their antimicrobial, physicomechanical and chemical properties. **(Paper I)**
2. Explore the effect of CHX on cell viability, antimicrobial, physical and chemical properties of endodontic sealers' leachates. **(Paper III)**
3. Assess the effect of interactions between sealers and irrigation regimes on the dentine-to-sealer interface in an *ex vivo* tooth model on the antimicrobial properties of the adjoining surfaces. **(Paper II)**
4. Assess whether NaOCl or CHX and two irrigation protocols may alter the antibacterial properties of dentine and three endodontic sealers. (**Paper II**)

Summary of the results

In **Paper I**, the sealer surfaces after contact with CHX were tested for antimicrobial and physicochemical properties as well as material characterisation was performed. Contact with 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 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 in contact with CHX was hydrophobic. The microhardness of sealers was reduced and the surface roughness increased after CHX exposure for AH Plus and BioRoot RCS, but decreased for PCS. CHX did not affect the sealers' chemical constitution, but PCS exhibited two extra phases.

In **Paper II**, an ex vivo tooth model was developed in order to investigate the residual antimicrobial effect of dentin and sealer after exposure to NaOCl or CHX and applied according to two established irrigation protocols. The model was considered reproducible as SEM examination of dentine samples indicated consistent separation between dentine and sealer surfaces. Residual CHX and irrigation protocol with CHX enhanced the antibacterial properties of dentine without sealer application, as well as dentine in contact with all three sealers tested, especially against planktonic *E. faecalis*. CHX also improved the antibacterial effect of AH Plus surfaces for all three incubation times. No irrigation groups or irrigation protocols altered the antibacterial properties of BioRoot RCS surfaces against planktonic bacteria or biofilms. Only BioRoot RCS surfaces eliminated the planktonic *E. faecalis* in all irrigation groups and protocols investigated, while PCS surfaces eliminate *E. faecalis* in biofilms for up to 7 days.

In **Paper III**, the sealer leachates after contact with CHX were tested for antimicrobial activity, cell viability and physicochemical properties. Exposure to 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, which was decreased. CHX contact did not change the sealers' alkalinity in distilled water."

Chapter 3

Methodological aspects

The biological (antimicrobial activity and cytotoxicity), the physicomechanical (setting time, wettability, microhardness, surface roughness, water uptake, sorption, solubility, porosity) and the chemical (material characterization and pH assessment) properties of the sealers were evaluated after exposure to CHX. This chapter aims to describe the methods and discuss why these were chosen over other complementary techniques.

3.1 Materials

3.1.1 Endodontic Sealers

An epoxy resin-based sealer, AH Plus (Dentsply International Inc, York, PA, USA), 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. Sealers with distinctly different chemical compositions were selected to assess chemistry's role in the interactions between the sealers and irrigation solutions. Different ageing periods were tested (up to 28 days) to determine the role of the setting in sealers' properties.

AH Plus BioRoot™ RCS Pulp Canal Sealer

Figure 3.1: Endodontic sealers tested in this thesis: AH Plus; BioRoot RCS; Pulp Canal Sealer

3.1.2 Irrigation Solutions

The following irrigation liquids were used: 1% NaOCl (Lot # 13678, Nordenta, Hørning, Denmark), 2% CHX (20% in water diluted and standardised to 2%, Lot # BCBS7878V, Sigma-Aldrich, St. Louis, MO, USA), 17% EDTA (Lot # 19120, Pulpdent, Watertown, MA, USA). Different irrigation protocols have been employed in endodontics using NaOCl, CHX and EDTA in different sequences and concentrations. CHX was chosen for more thorough testing as it is suggested mainly as a last irrigant: it may therefore affect the properties of dentine and subsequently the properties of the sealers placed after the chemomechanical preparation of the root canal.

3.2 Study design

The main scope of the present thesis was to investigate the effects of CHX on sealers' surfaces and leachates (Paper I & III). Thus, we applied CHX directly on the sealers' surfaces. In addition, the sealer surfaces were exposed to saline pre-treatment prior to testing against planktonic bacteria as a first approach to investigate whether the presence of moisture might affect the antibacterial activity of the materials (Paper I) [282]. Human teeth and training blocks (Endo-Training-Bloc, Dentsply Maillefer, Ballaigues, Switzerland) were used to simulate clinical application of the sealers after a final irrigation with CHX or saline (Paper I). An *ex-vivo* tooth model was further used to assess the

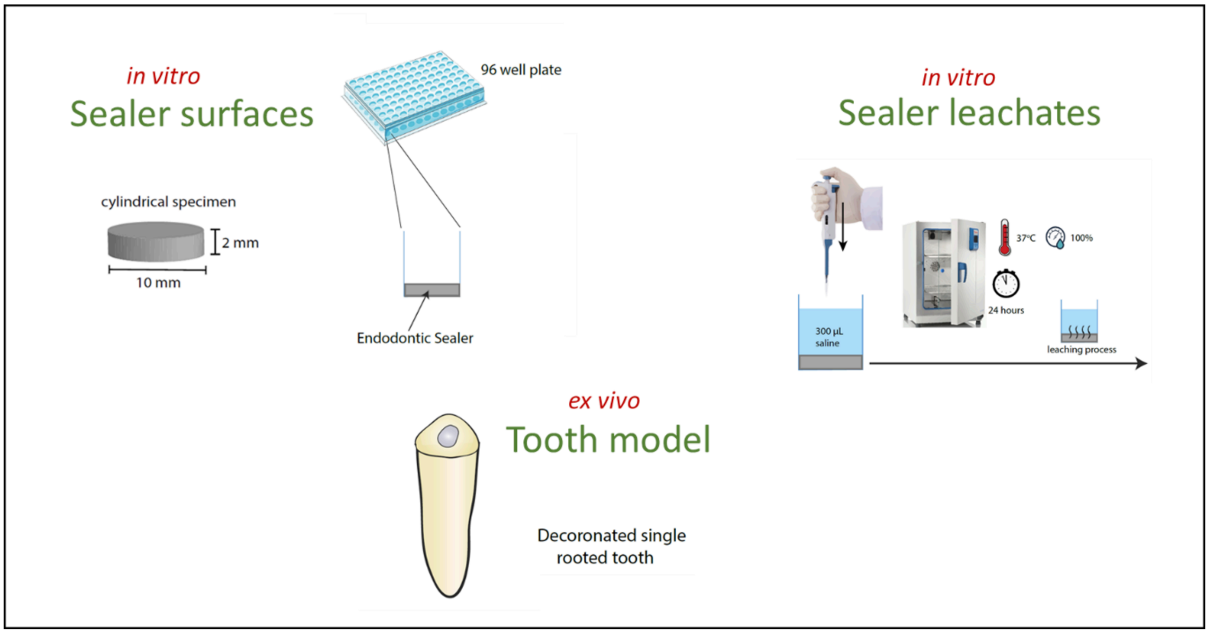

Figure 3.2: Schematic representation of the study design. Both surfaces and leachates of the endodontic sealers were tested. An *ex-vivo* tooth model was also developed for material characterization and to test the residual effect of NaOCl and CHX as well as two irrigation regimes.

modulation of antimicrobial properties by irrigation (Paper II) (Figure 3.2). The purpose of using a tooth model was to elucidate the role of dentine in the interactions between CHX and sealers according to their chemistry.

In Paper III the leachates of the sealers were investigated. Contact between tissue fluids or irrigation liquids and sealer may cause the leaching of constituents from the sealer. Leachates could potentially migrate to patent dentinal tubules, lateral canals, or periapical tissues through the bulk of filling materials or the dentine-sealer interface [153, 271, 272, 355]. Leachates of endodontic materials have attracted attention in regard to antibacterial properties and cytotoxicity [356]. The antibacterial properties of leachates may aid in eradicating residual planktonic bacteria or bacteria in biofilms in untouched areas after chemo-mechanical preparation [28, 351, 357-362]. At the same time, the leachable compounds should ideally not be cytotoxic to the periapical tissues as this may retard the healing process and thus jeopardise the clinical success of root canal therapies [264, 363].

CHX application (Paper I & III)

The amount of CHX applied to the surface of each sealer specimen for testing antimicrobial and physical properties was standardised. 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 [364]. Moreover, the three sealers tested in our studies 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 distributed with a sterile plastic inoculation loop. In addition, different devices were used to test antibacterial and physical properties. 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 goal was to apply as little CHX as possible in order to

sufficiently cover the surface area of the sealers and imitate the clinical scenario. Considering 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 to cover the samples' whole surface area.

3.3 Sealer surfaces and leachates

3.3.1 Biological properties

Antimicrobial properties

Direct contact tests (DCTs) were employed for antimicrobial testing for both planktonic bacteria and bacteria in biofilms (Figure 3.3). The antimicrobial properties of both sealer

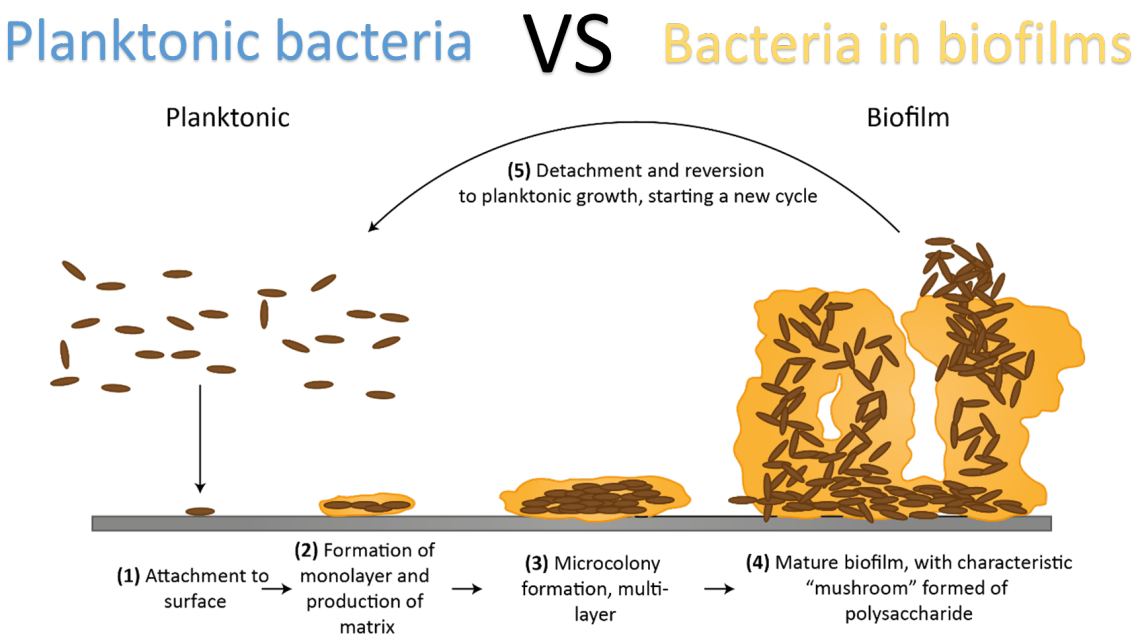

Figure 3.3: Schematic representation of planktonic bacteria and bacteria in biofilms. Retrieved from: https://www.immunology.org/public-information/bitesized-immunology/pathogens-disease/biofilms-and-their-role-pathogenesis

surfaces (Paper I & II), dentine (Paper II) and sealer leachates (Paper III) were tested with the use of direct contact tests. The DCT has been preferred to the classic agar diffusion test (ADT) to overcome the latter's limitations: semiquantitative nature, limitation to distinguish between bacteriostatic and bactericidal activity, and inability to detect the activity of insoluble components [365-367]. Statistical analysis was performed on the CFUs calculation, which constitutes a well-documented method to quantify the bactericidal effect of antimicrobials [282, 365]. Thus, a quantitative tool based on microbiological culturing (the plate count method, CFUs counts) was chosen to assess bacterial viability. The CFUs counts is a widely used technique and readily available in most microbiological labs. It is a reproducible

technique that enables comparisons between experiments. According to a recent systematic review to identify articles employing a laboratory endodontic biofilm model [368], in 85% (66/77) of the studies, microbiological culturing was used as one or the sole evaluation method.

CFUs counts of bacteria in biofilms require mechanical detachment of bacteria from the surfaces they have been in contact with. Comparisons of different methods have been well reviewed; however, there is no universally accepted gold standard for the quantification of bacteria in biofilm [369]. Two prerequisites for an ideal processing method should be met: to retrieve as many bacteria as possible and not adversely affect bacterial viability. A balance should be achieved to ensure both maximal retrieval and maximal viability of bacteria. Short-time vortexing is widely used and preferred for young biofilms, whereas it may not be enough to retrieve the large cell clusters formed in older biofilms [370]. In our study, young 48 hours biofilms were grown, and thus vortexing was favoured over other techniques such as sonication.

Selection of bacteria

Enterococcus faecalis American Type Cell Culture Collection (ATCC) 19434, *Streptococcus mutans* ATCC 700610, *Staphylococcus epidermidis* ATCC 35984 and Staphylococcus aureus Newman were used for the antibacterial properties against sealer surfaces *in vitro* (Paper I) and sealer leachates (Paper III). For testing sealer and dentine surfaces in the *ex-vivo* tooth model, *Enterococcus faecalis* ATCC 19434 was used. In endodontic infections, the root canals can be hosted by planktonic bacteria and bacteria in biofilms, on dentin walls and into dentinal tubuli [28, 30, 361, 362]. After chemomechanical preparation, residual planktonic bacteria or biofilms can remain in remote areas such as apical ramifications, lateral canals, and isthmuses [28, 351, 357-360]. In this study, sealers' antibacterial properties were assessed against planktonic bacteria and bacteria in monospecies biofilms. *E. faecalis*, *S. epidermidis* and *S. aureus* have been associated with post-treatment apical periodontitis [92, 371, 372]. *S. mutans*, a pathogen associated with caries, has also been reported in necrotic root canals [67, 373], and it was included in the present study as a reference to evaluate the susceptibility of species not commonly retrieved from such infections [282]. 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 [374]. Moreover, the tested microorganisms are robust and easy to cultivate in the laboratory. Regarding *E. faecalis*, numerous *in vitro* and *ex vivo* studies have used this bacterium to test the antibacterial properties of endodontic materials [349, 350, 368]. Thus, the use of *E. faecalis* enabled comparisons with other studies in the literature. Furthermore, *E. faecalis* can colonize dentine and form biofilms on different substrates, including root canal-filling materials [375, 376].

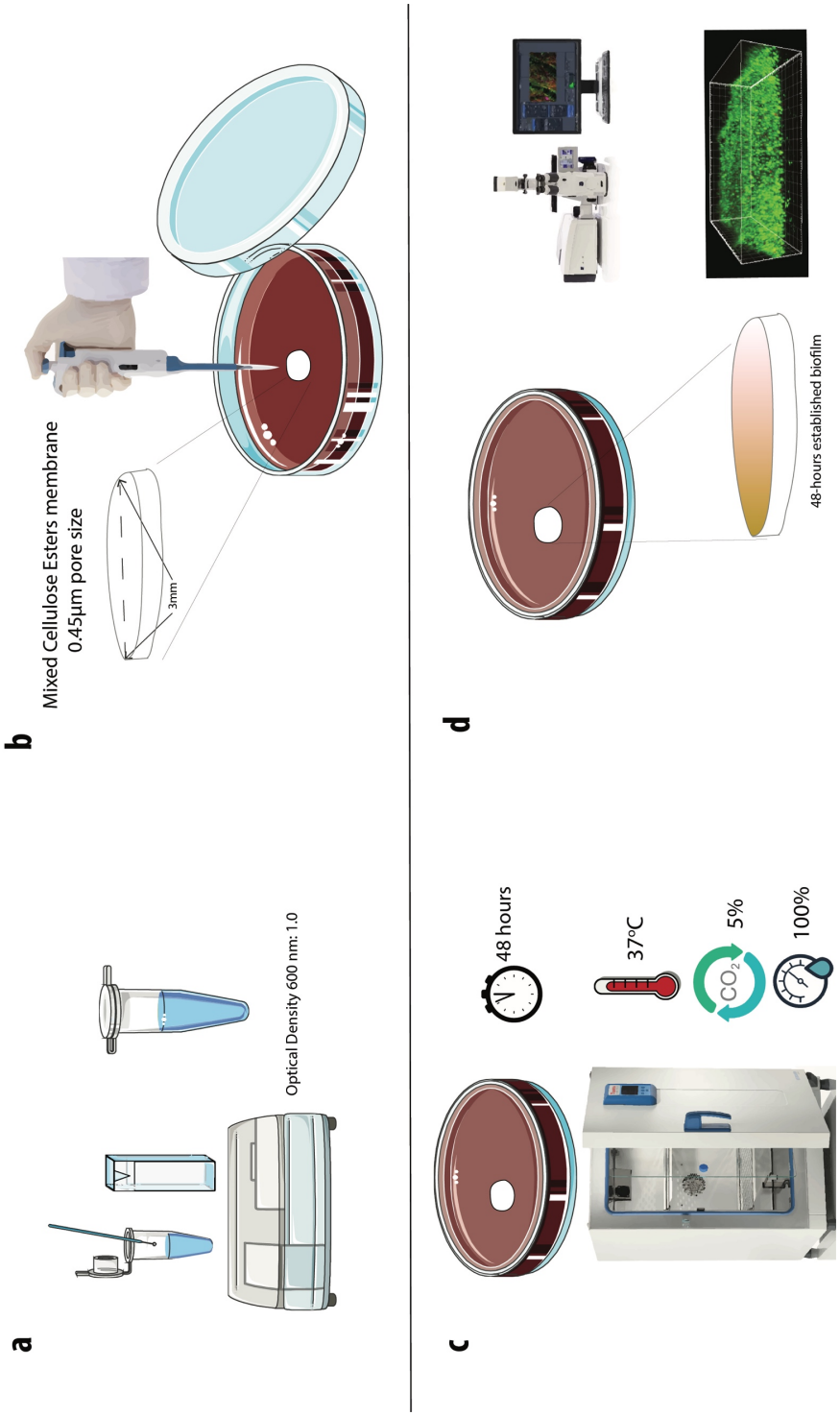

Figure 3.4: Schematic representation of biofilm formation. The bacteria were suspended in PBS to an optical density at 600 nanometers (OD600) of 1.0 (a). Membrane filters were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 µl of each bacterial inoculum OD600 1.0 was applied upon the outer surface of membranes (b). The agar plates were incubated at 37°C in a 5% CO_2 supplemented atmosphere for 48 hours (c). The monospecies biofilms were established and verified with the use of confocal laser scanning microscopy (d)

Bacteria in biofilms

Two biofilm models were developed to assess the antibacterial properties of the sealer surfaces (Figure 3.4) and leachates (Figure 3.6). For sealer surfaces (paper I & II), biofilms were formed upon membrane filters (MF-Millipore™ Membrane Filter, 0.45 µm pore, Merck, Darmstadt, Germany) placed upon TSB agar plates. For sealer leachates (paper III), biofilms were formed upon polyester coverslip discs (13 mm, Nunc™ Thermanox™ Coverslips, Thermo Fisher Scientific, Waltham, MA, USA) that 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 hours in TSB were mixed with fresh medium at a fixed rate of 1/10.

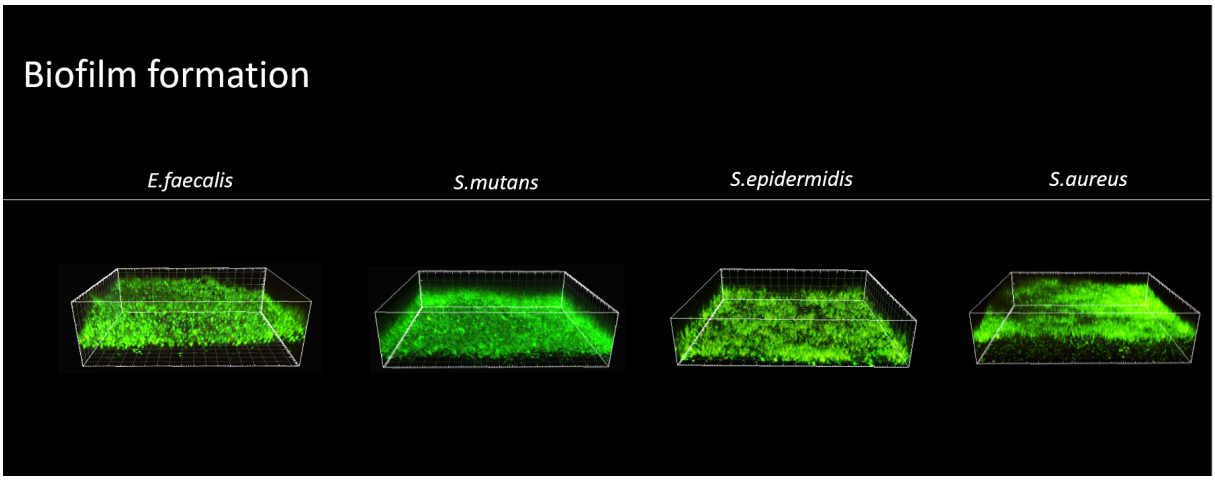

Figure 3.5: Indicative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-hours monospecies biofilms grown on membrane filters.

Two ml of each bacterial suspension were transferred into the 24-well plates and covered the coverslip discs sufficiently. Both biofilm models were verified for biofilm formation with the use of confocal laser scanning microscopy (CLSM; Olympus FluoView FV1200, Olympus Corp, Tokyo, Japan) (Figures 3.5 and 3.7). Possible carryover effect was measured for both biofilm models. A factor to consider is the maturation stage of biofilms, as it is well-documented that young biofilms are more susceptible to antimicrobial agents than mature ones [377, 378]. Concerning the choice of substrates, bovine dentin or human dentin were the ones to grow *E. faecalis* biofilms in previous studies [351, 366]. However, possible carryover effect or partial retrieval of bacteria can occur as the tested material may firmly adhere to dentin [282]. Due to high hydrophilicity of MCE, the membranes enabled the biofilm-sealer separation process, minimising biofilms' disruption. For testing sealer leachates, the resilient polyester coverslip discs were favoured to withstand contact with the liquid. Regarding contact between bacteria and sealers, short contact time may be fallacious about the antibacterial activity of the sealers against biofilms, while sealers maintain their antibacterial efficacy throughout the setting [282]. To address this, the antimicrobial properties of sealer surfaces were tested against established biofilms for 24 hours.

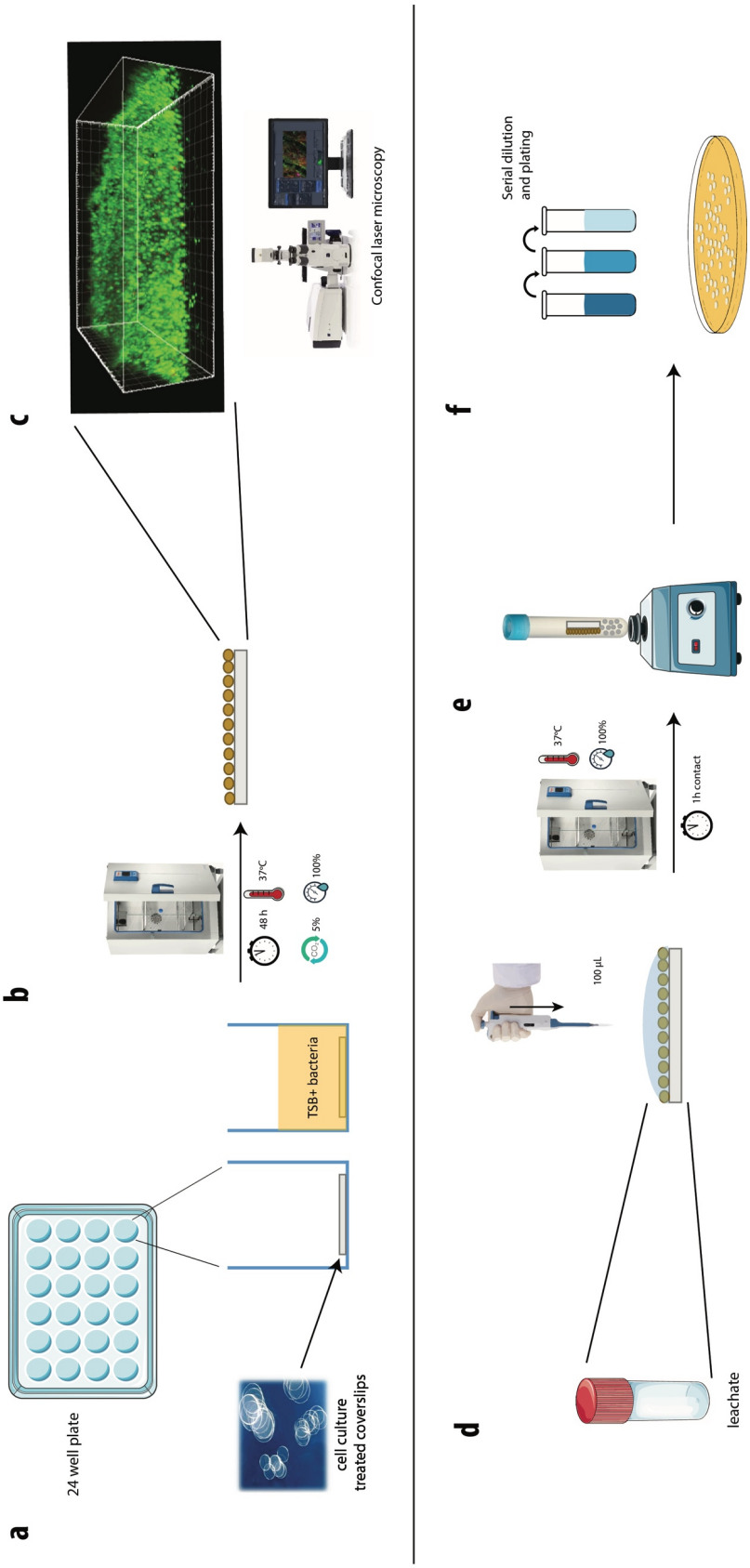

Figure 3.6: Schematic representation of antibacterial assay for bacteria in biofilms. Polyester coverslip discs were placed on the bottoms of 24-well plates. Bacteria grown overnight in TSB were mixed with fresh medium in a fixed rate 1/10. Two ml of each bacterial suspension were transferred into the 24-well plates and covered sufficiently the coverslip discs (a). The plates were incubated at 37°C in a 5% CO_2 supplemented atmosphere for 48 hours and monospecies biofilms were established (b). Confocal laser-scanning microscopy (c). A hundred µl of each leachate were applied on the discs for 1 hour at 37°C in contact with the biofilms (d). After contact time, each disc was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads (e). After serial dilutions in PBS, CFUs were counted after overnight incubation at 37°C in a 5% CO_2 supplemented atmosphere (f)

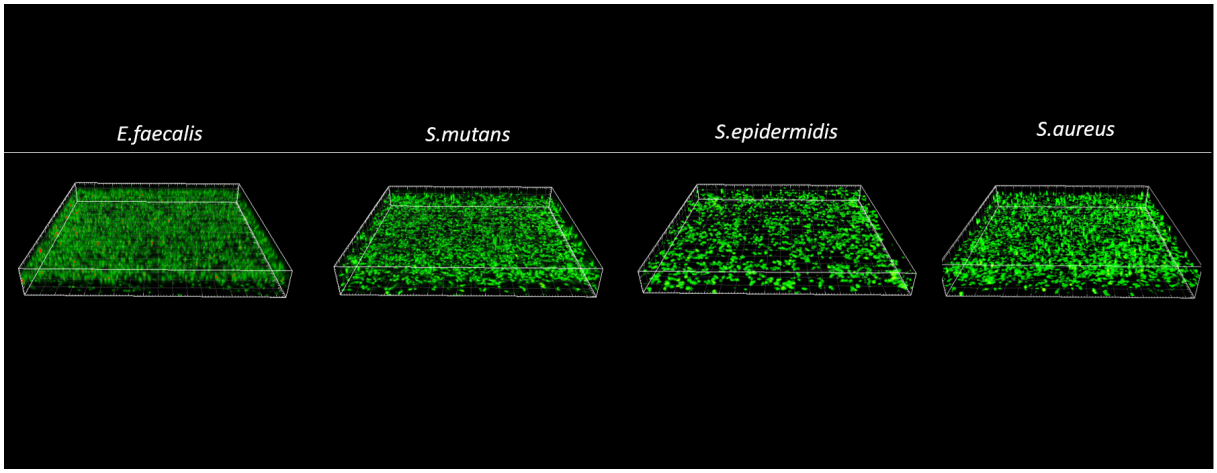

Figure 3.7: Representative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-hours monospecies biofilms grown on polyester coverslips.

Cytotoxicity-cell viability (Paper III)

Regarding cytotoxicity, many different methods have been applied in the literature assessing various parameters such as cell viability, proliferation, apoptosis, adhesion, morphology, and gene expression. In our study, the cytotoxicity of sealer leachates was evaluated with the use of MTT assay, which is widely used to assess the cell viability of such materials [270, 379, 380]. It is a standardised method and a reliable indicator of cellular metabolic activity [381]. As sealer leachates with and without CHX contact were tested herein for the first time, a highly reproducible and trustworthy technique such as MTT was favoured. Leachates from freshly mixed, 24 hours, 7 days and 28 days set sealers with and without 1-minute contact with CHX were tested and L929 murine fibroblast cell line was chosen. For negative controls, mixture of saline with cell culture medium in a 1:1 ratio was transferred upon the seeded cells. For positive controls, CHX in different dilutions was used. The 3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma M2128) was employed to evaluate cell metabolic function [382].

3.3.2 Physicomechanical properties

To secure a hermetic seal, endodontic sealers should ideally be inert and keep long-term dimensional and physicochemical stability inside the root canal space. Nevertheless, it has been shown that antimicrobial additives can alter the properties of dental materials and impair their clinical performance [383, 384]. It is paramount to ensure that antimicrobial agents in conjunction with sealers may favour the antimicrobial action without altering their physicomechanical properties and chemical constitution [288].

Physicomechanical properties (Paper I)

Setting time (ISO 6876), wettability, microhardness, and surface roughness of the sealers alone or in contact with CHX and water or HBSS (surface roughness) were evaluated. 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 [385].

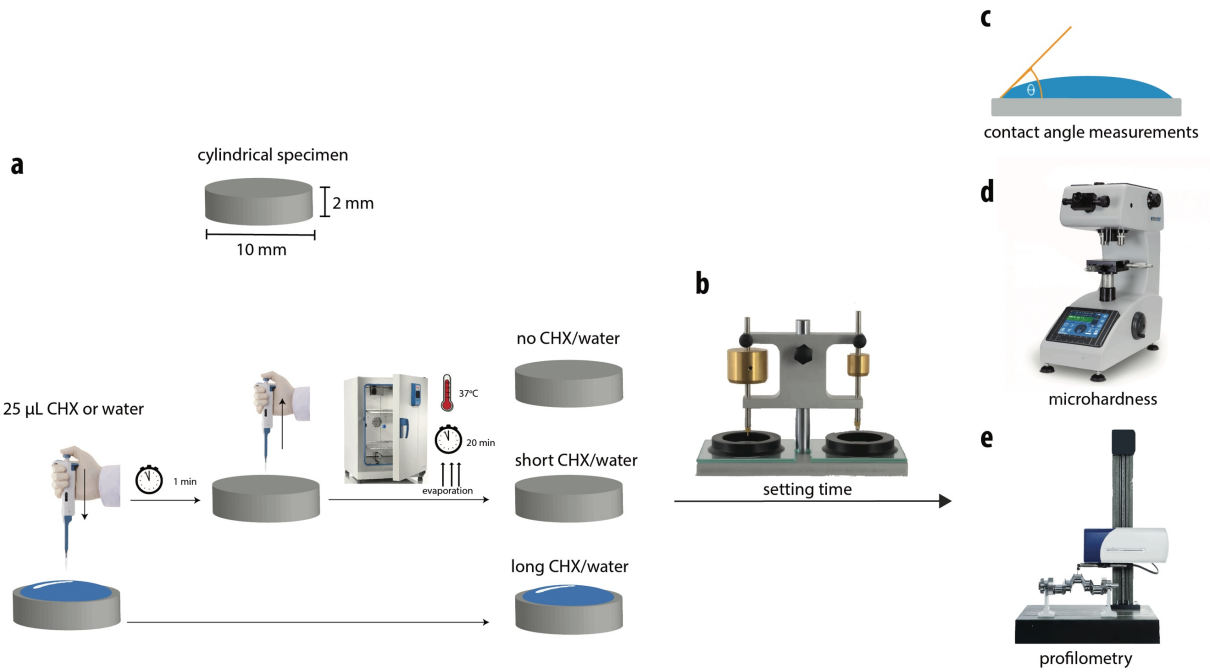

Figure 3.7: Cylindrical specimens were prepared into moulds for each sealer, measuring 10 mm in diameter and 2 mm in height. For both CHX and water exposure groups, a drop of 25 µl was applied upon the sealers with a pipette(a). The physical properties were assessed by testing the setting time (b), wettability (contact angle measurements) (c), microhardness (d), and surface roughness of sealers (e).

Setting time, wettability (contact angle measurements), microhardness, and surface roughness of sealers were tested as follows: Cylindrical specimens, measuring 10 mm in diameter and 2 mm in height, were prepared into moulds for each sealer. For setting time, wettability and microhardness tests, after preparation, the sealers were incubated in a 100% humidified atmosphere at 37°C and allowed to set for 24 hours with and without contact with 2% CHX or water. For the surface roughness test, in addition to no contact and CHX groups, measurements of the samples were also taken for 24 hours of contact with Hanks' Balanced Salt Solution (HBSS; Sigma Aldrich, Gillingham UK) (Figure 3.7).

Setting time

The prolonged setting of a sealer increases the possibility of contacting periodontium tissue and imposing cytotoxic effects [380]. As for antimicrobial properties, the majority of the sealers exhibit short-term effectiveness, which is compromised after setting [350]. Furthermore, longer setting times might permit more extensive interactions between sealers and possible contaminating substances as the irrigants [386]. The slow setting time of a sealer may be connected with high washout and disintegration that may jeopardise the sealing ability and integrity of a root canal filling [387, 388]. Furthermore, longer setting times might permit more extensive interactions between sealers and irrigants [386]. The setting times of the sealers were analysed in compliance with ISO 6876 (2012) [389]. It has been shown the influence of the environment on the setting of endodontic sealers [311]. Moreover, in the clinical scenario, the setting of the sealers may differ in comparison with *in vitro* conditions [390]. However, the Gilmore technique indicated by ISO 6876 remains the technique of choice for evaluating setting time regardless of the testing conditions and the effect of the environment. A stopwatch was started after preparing the sealers and placing 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 the setting. The 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.

Contact angle measurements

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. The lower the contact angle, the faster the liquid will spread on substrates and wet the surface (hydrophilicity) [391]**.** This could potentially have affected the DCT assays where bacterial suspensions were applied as drops on sealer surfaces. A cutoff on the 90 degrees has been accepted to define hydrophobicity (θ > 90°) and hydrophilicity (θ < 90°) of materials' surfaces [392]. It has been shown that the 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 [281, 338, 340], which may indirectly affect their antibacterial efficacy. Contact angle measurement was used to investigate the wettability of the material surfaces. A 20-µL 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).

Microhardness test

Microhardness of a material is a measure of multiple properties. It can be used as an indicator of the setting process and to show how different setting conditions can affect the overall surface strength of materials [393]. The low microhardness of dental materials has been connected to reduced bond strength to dentine and sealing ability [394]. Microhardness testing was carried out by applying an indentation technique using a hardness-testing instrument (Struers A/S, Rødevre, 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. The Knoop hardness test, also referred to as a microhardness test method, uses a more elongated or rectangular shape indenter and is mainly employed for small parts and thin sections such as foils. The width of the Knoop indentation can provide more resolution for measurement, and the indentation is also less deep. Since the test indentation is minimal in a Knoop test, the Vickers microhardness method was favoured.

Assessment of surface roughness

The surface roughness of substrates has been related to initial bacterial adhesion during biofilm formation [255]. Surface analysis of the samples was carried out using mechanical profilometry (Form Talysurf Series 2, Taylor Hobson, Leicester, 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 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×). Apart from using a stylus (mechanical profilometer), optical methods are increasingly used to measure surface roughness (optical profilometer). Moreover, SEM stereoscopy has been used for the assessment of surface roughness. Briefly, backscattered images are obtained at independent sections of each sealer specimen with 4 different tilt angles at a specific magnification. Stereoscopic reconstruction in a 3D model of these images is performed using suitable software, and the surface roughness values are calculated following calibration of the programme based on a reference angle (60°) artificially induced upon the surface of each sealer. Each of the techniques above has its advantages and disadvantages. Mechanical profilometry is a technique where the stylus (profilometer) touches the specimens and might produce scratches on their surfaces. In our study, this was not an issue since the sealer specimen were explicitly manufactured for the surface roughness measurements and were not used in other assays. Its main advantage is that it is a non-expensive technique with high reproducibility. Optical profilometry is a non-destructive technique that images an area of the surface without touching the specimens. However, optical methods, including SEM stereoscopy, are sensitive to several surface qualities besides surface height. These include optical constants, surface slopes, fine surface

features that cause diffraction, and deep valleys in which multiple scattering may occur. In addition, scattering from surfaces within the optical system produces stray light that can affect the accuracy of an optical profiling method [395].

Physicomechanical properties (Paper III)

Water uptake, sorption, solubility, and porosity of sealers with and without CHX contact were evaluated following a modification of ISO 4049; 2019 [396] regarding the manufacturing of sealer specimens. Normally in ISO 4049, specimens measuring 15 mm in diameter and 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) were manufactured in such a way as 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, 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 (Figure 3.8).

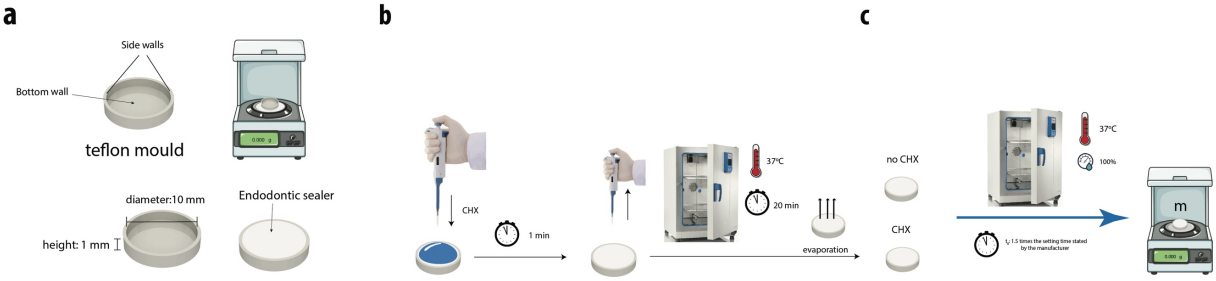

Figure 3.8: Schematic representation of sealer preparation for ISO 4049. The sealers were placed into the moulds (a) and were either allowed to set independently (no CHX) or in contact with CHX. After 1 minute of contact with CHX, the drop was removed with a pipette (b), and the sealers were placed in a dry incubator at 37°C for 20 minutes to let any excess liquid dry out before being allowed to set (c).

ISO 4049

It is also important that irrigation solutions favour the biological properties of sealers without altering their physicomechanical behaviour and chemical constitution. In the present study, ISO 4049 was selected to be performed as it allows the assessment of various parameters (water uptake, sorption, solubility) with the same study design. These properties were important to be tested as hydraulic calcium silicate-based cements, such as BioRoot RCS, present high hydrophilicity of their surfaces which in turn leads to increased water adsorption and porosity. Moreover, its hydraulic nature and the formation of calcium hydroxide render the sealer susceptible to environmental conditions [311]. Our adjusted ISO 4049 study design further enables the evaluation of porosity based on a previously described

gravimetric method [397] and the pH measurement of the soaking (immersion) liquids. Porosity was investigated as, besides poor physical properties, open pores in the bulk of endodontic sealers may serve as hubs and favour bacterial growth [398]. Moreover, nutrients entering the root canal may find pathways through the bulk of filling materials via pores and facilitate the growth of entombed bacteria [399, 400]. 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) suggests 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 sealers' physical properties, focusing on the leaching of the sealer surfaces in contact with CHX. Inert teflon cylindrical moulds (with bottom and side walls) 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.

A few studies have assessed the leaching of sealers and characterized their leachates [271, 308, 311, 401]. BioRoot RCS does not comply with ISO 6876 as it interacts with the environment in which it is placed. The ISO 6876 tests material properties when immersed in water. This applies to materials that are not water-based and inert such as the AH Plus and zinc oxide eugenol-based sealers, as is the Pulp Canal Sealer. The solubility based on the ISO 6876 uses 2 large sealer discs placed in water for 24 hours. The solubility is calculated by measuring the amount of material lost from the disc when the fluid is evaporated rather than the weight of the disc itself. Instead of water, the use of simulated body fluids to immerse the BioRoot RCS in an appropriate environment similar to the clinical situation has shown a very different value for solubility. This indicates that this ISO 6876 method is not suitable for hydraulic cements. This has been published and explained in a recent publication [311].

The paper III puts forward a methodology where contact with sealers and irrigating solutions is assessed. This contact is direct on the material, and thus methods testing the physical properties on the level of interaction between material surfaces and irrigating solutions were adopted. The aim was to examine the physical properties of the sealers focusing on the leaching of the sealer surfaces in contact with CHX. Inert teflon cylindrical moulds (with bottom and side walls) were manufactured in such a way as 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.

Since the ISO 6876 tests the solution rather than the discs, the ISO 4049 was a neat choice as this standard enables the measurement of the sorption, solubility and porosity using the same specimens and is also tested directly on the material. Although this standard is meant to be used to test resin-based restorative materials, its methodology was very suitable for the tests undertaken in this study.

The misuse of standards has been brought up in a recent editorial [402], where the ISO 4049 was also specifically discussed. ISO standards are not research tools but rather a method of certification. In this context, we have not used the ISO 4049 as a standard to certify materials, which in fact, is not applicable since the materials are sealers but rather that the test was convenient and tied up well with the rest of the tests undertaken. ISO 4049 was used to assess specific material properties.

3.3.4 Microscopic evaluation of sealer surfaces (Paper III)

Optical microscopy (NexiousZoom, Euromex, Arnhem, The Nederlands) 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 was aforementioned, incubated at 37^{o}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).

3.3.5 Chemical properties - Assessment of pH (Paper III)

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

3.4 Tooth models (Paper I & II)

An *ex-vivo* tooth model was also developed for material characterisation (Paper I) and another to test the residual effect of NaOCl and CHX after irrigation as well as two irrigation regimes (Paper II).

3.4.1 Material characterization (Paper I)

A split tooth model was used to simulate a clinical setting with irrigation of CHX as the last irrigant (Ethical approval REC Ref 14/EM/1128: Research and Innovation Department, Birmingham Community Healthcare, NHS). Complementary to the tooth model endo-training blocks (Endo-Training-Bloc, Dentsply Maillefer, Ballaigues, Switzerland) were also used. The aim in employing both a tooth model and endo training blocks was twofold: firstly, to replicate realistic clinical conditions, and secondly, to scrutinize the influence of dentin as a substrate on the intricate interactions between CHX and sealers. Dentin has been reported to affect

material properties, both irrigation solutions and endodontic sealers and vice versa [337, 351]. The exposed sealers upon their substrates (either tooth or endo training blocks) were characterised 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 3.9).

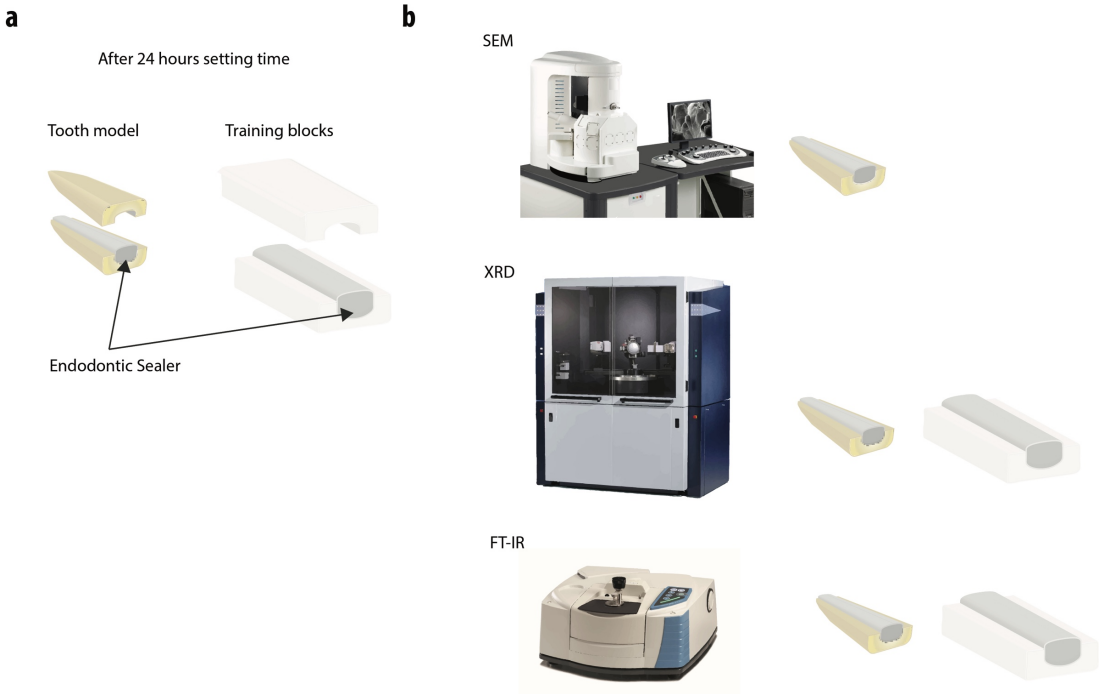

Figure 3.9: Assessment of chemical properties. After the setting period, each root was unwrapped from the Parafilm M, and the root fragments were gently detached using a scalpel that was applied on the thin space between them (a). The sealers were exposed and gently retrieved intact from the dentine walls. The preparation was also applied on endo-training resin blocks. The exposed sealers upon their substrates (either tooth or endo training blocks) were characterised by scanning electron microscopy (SEM) and energy dispersive spectroscopy, X-ray diffraction analysis (XRD), and Fourier-transform infrared spectroscopy (FTIR) (b).

Scanning Electron Microscopic examination-Elemental analysis

SEM examination was performed on sealer-tooth samples. SEM analysis provided detailed information on the elemental constitution of the sealers in tooth model and microstructural characteristics of their surfaces under short and long exposure to CHX. These were mounted on aluminium 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 surface of the intact sealer.

X-ray Diffraction analysis

XRD is useful for extracting information on the crystallographic structure and can be used to identify the phase composition of solids. 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 analysed as controls. The surface analysis was performed using glancing angle X-ray diffraction analysis at a fixed angle of incidence of 5. Phase identification was accomplished using a search-match software utilising Crystallography Open Database (COD) (Diffrac.Eva, Bruker, Billerica, Massachusetts, MA, USA).

Fourier Transform Infrared spectroscopy (FT-IR)

The Fourier transform infrared spectroscopy assesses the chemical changes in the sealer after contact with CHX. Through IR spectroscopy, it is possible to determine the functional groups of the chemical substances and identify any changes in the chemical structure of the materials. 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 analysed by stacking the spectra retrieved from the FT-IR, using the accompanied software (Omnic).

3.4.2 Antimicrobial properties (Paper II)

Preparation of root blocks

Extracted human teeth were collected from a bio-bank ("2013/413 NIOM tannbank") approved by the Regional Committees for Medical and Health Research Ethics (REC, application number 28748), Norway. All teeth were decoronated, and their roots were horizontally sectioned at the apical parts at a level to form root blocks with a standardised length of 7 mm, using a precision cutting machine (Buehler 11-1280-160 Isomet Low Speed Saw, Buehler, Lake Bluff, IL, USA). The roots were instrumented with ProTaper rotary files (Dentsply Maillefer, Ballaigues, Switzerland) up to size F4, and further enlarged with fiber post drill (3M Relyx Fiber Post Drill No 3, 3M, St. Paul, MN, USA). Oval-shape root canals were prepared measuring approximately 4 mm at the largest diameter (semi-major axis). Irrigation with 2 mL of 1% NaOCl was followed between the changes of the rotary files and a last rinse with 0.9% saline using a 27 gauge Monoject 3cc Endodontic Syringe (CardinalHealth, Dublin, Ireland). The root blocks were further segmented (dichotomised) vertically with the use of the diamond saw and the two segments were repositioned and held tightly together by wrapping them up with the use of Parafilm M (Bemis Inc, Neenah, WI, USA) (Figure 3.10).

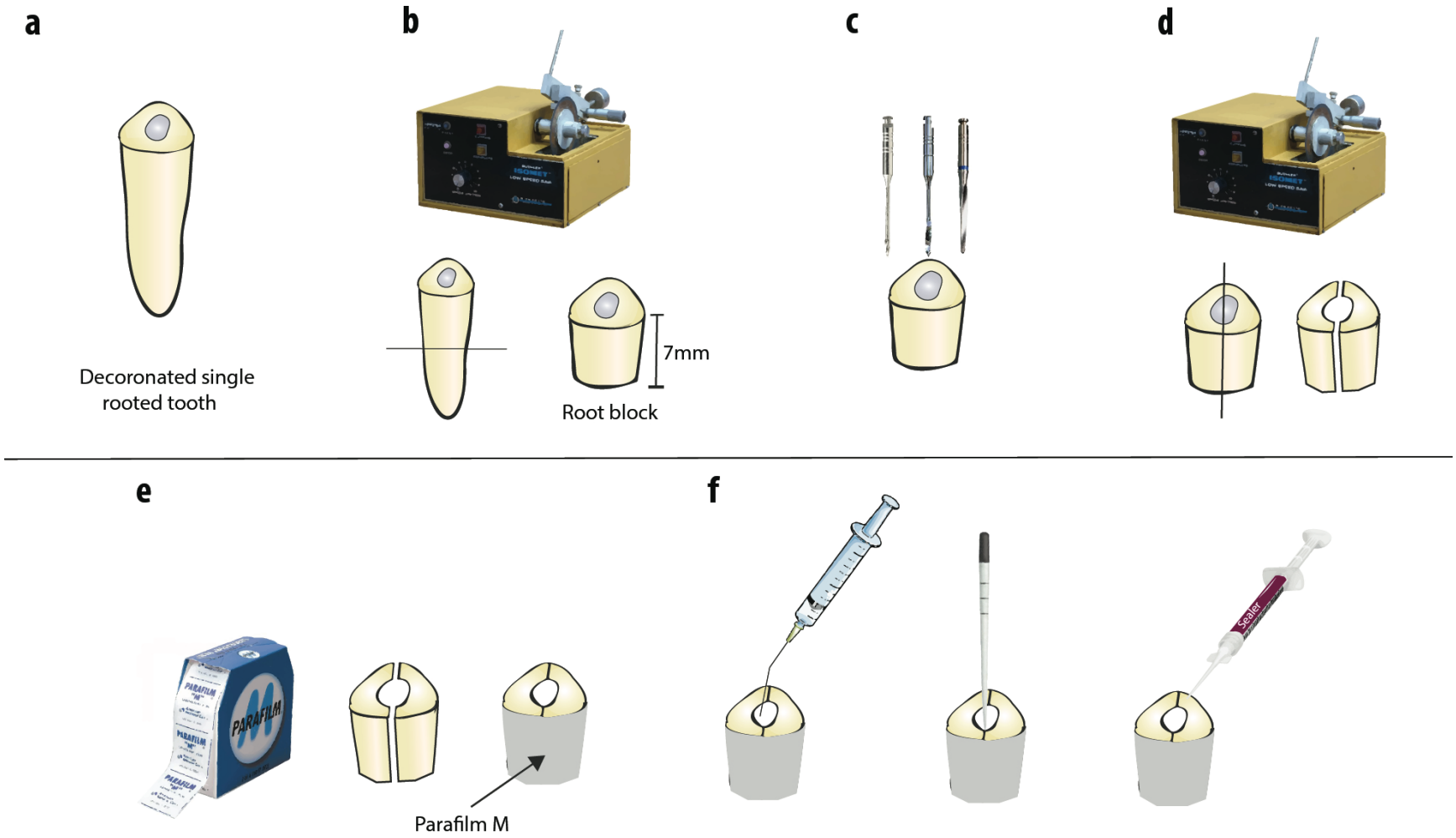

Figure 3.10: Schematic representation of teeth preparation. All teeth were decoronated (a) and their roots were horizontally sectioned at the apical parts, at a level to form root blocks with a standardised length of 7 mm, using a precision cutting machine (b). The root canals were instrumented with rotary files and further enlarged with fibre post drill (c). The roots were further segmented (dichotomised) vertically with the use of the diamond saw (d) and the two segments were repositioned and held tightly together by wrapping them up with the use of Parafilm M (e). After irrigation, the tested sealers were mixed according to the manufacturer's instructions and placed inside the root canal-training resin blocks.

Validation of split tooth model-Evaluation of dentine surfaces

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

To assess the type of failure on the sealer-dentine interface (adhesive: complete separation of sealer from dentine, cohesive: rupture of material bulk within the sealer, or a mix) and identify any sealer remnants on dentinal walls, scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) were performed. Briefly, two root blocks for each sealer were mounted on aluminium stubs, carbon coated (Agar Scientific, Stansted, UK), and viewed with the scanning electron microscope (TM4000Plus, Hitachi, Tokyo, Japan). 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 selected spots and rectangular areas of the samples. Furthermore, elemental maps at the same levels were performed, and each element was marked out/ designated in a different colour. EDS was also conducted over sealers prepared in circular samples to define their elemental profile. At this point, it is emphasised that the analysis regards root blocks that were incubated for 24 hours, when the sealers were at the most premature stage of setting, compared to 7 and 28 days set materials and therefore were more prone to deform during the separation of tooth segments leading to a possible cohesive type of failure. Moreover, all root blocks were pretreated with 17% EDTA for 5 minutes aiming for the constant background of dentinal tubules, as the smear layer did not allow to distinguish between tooth structure and sealer remnants.

Elements that are traced both on sealer and tooth surfaces could not be indicative of sealer remnants on the dentine and were not evaluated. For example, the movement of calcium from the sealer to the tooth could not be monitored by the elemental mapping because both sealers and tooth structure contain calcium. Thus, those unique elements that could only be traced in sealers were guiding to identify the presence of sealer residues upon dentine (Figure 3.11): zirconium (Zr) and tungsten (W) for AH Plus; silicon (Si), chlorine (Cl) and Zr for BioRoot RCS; zinc (Zn) for PCS. SEM examination showed adhesive mode of failure at the sealer-dentine interface. Sealer residues could be sporadically identified, but no full dentine coverage was evident in any of the surfaces investigated. AH Plus bonded to dentine with sealer tags, and after the separation process, the whole bulk of the material was debonded. Only few sealer tags rich in Zr could be identified in dentinal tubules.

BioRoot RCS demonstrated trace elements on dentine without full coverage. As for PCS, the elemental analysis showed few sealer tags rich in Zn. Thus, the model was considered reproducible as the SEM examination of dentine samples indicated consistent separation between dentine and dentine-sealer surfaces. This finding enabled us to proceed further with antibacterial assays, testing both the dentine and dentine-sealer surfaces.

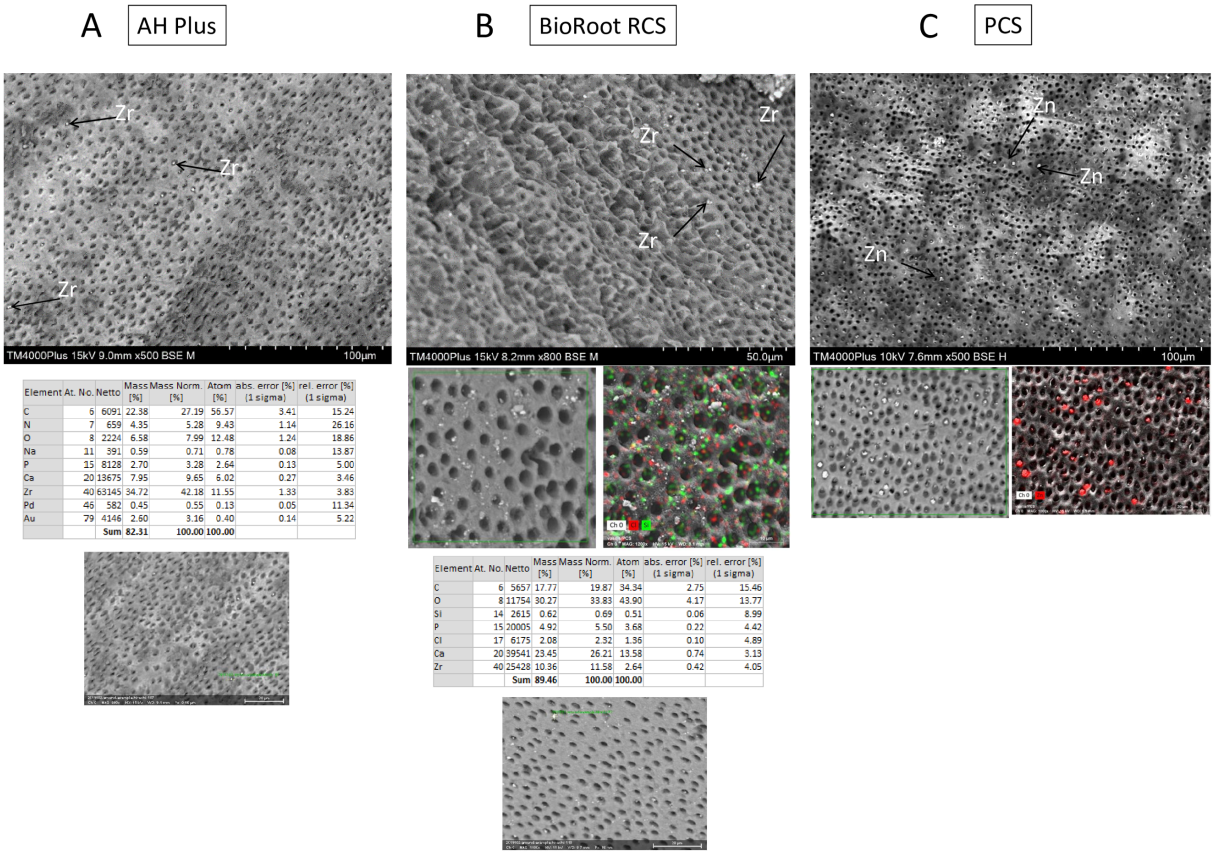

Figure 3.11: Representative scanning electron micrographs of dentine having been in contact with the tested sealers retrieved from the split tooth model: AH Plus (A), BioRoot RCS (B) and PCS (C). The black arrows indicate sealer residues (white circular spots) rich in Zr for AH Plus/BioRoot RCS and Zn for PCS, verified by elemental analysis. Elemental mapping of dentine in contact with BioRoot RCS shows the distribution of Cl and Si. Elemental mapping of dentine in contact with PCS indicates the presence of Zn.

Irrigation regimes and Obturation

The power calculation using G*Power 3.1 (Heinrich Heine University, Dusseldorf, Germany) to calculate the sample size of each experimental condition (both the residual effect of 1% NaOCl, 2% CHX and the antibacterial effect of two irrigation protocols, with and without sealer placement), indicated at least 7 root blocks in each assay (planktonic bacteria and bacteria in biofilms) (effect size f= 0.40, α error probability=0.05). Thus, 9 root blocks (n=9) were used for each experimental condition (Figure 3.12).

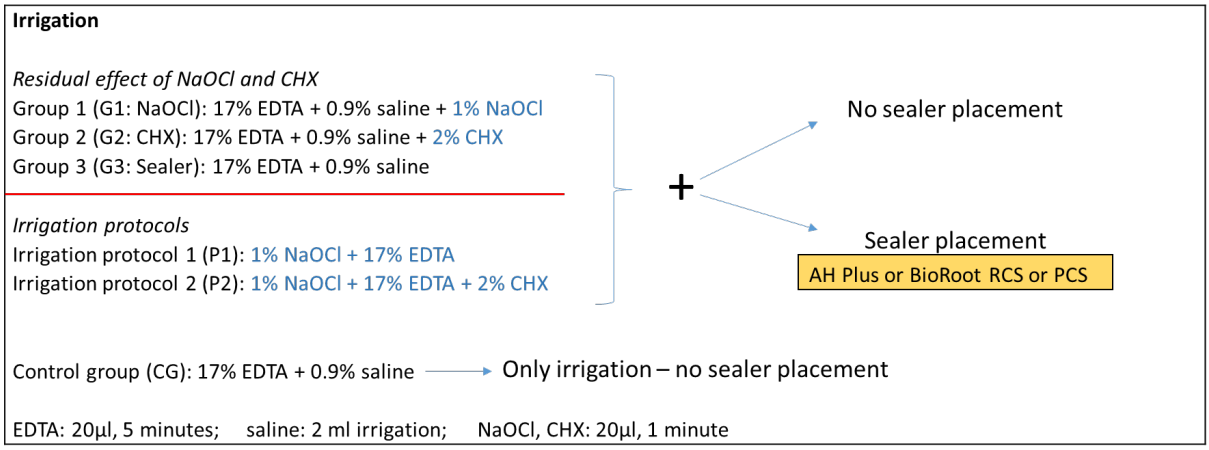

Figure 3.12. Sequence of irrigation liquids and their application time. Allocation of groups based on last irrigants and irrigation protocols.

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

Antibacterial assays

Planktonic bacteria-Direct Contact Test (DCT)

An amount of 5 µl from *E. faecalis* suspension was carefully placed upon the dentine (dentine sample) and the dentine-sealer surface (dentine-sealer sample) and only upon the dentine surface in irrigation groups without sealer placement and control group. The specimens were incubated at 37^{o}C for 1 hour while complete evaporation of the suspension's liquid was inspected (Figure 3.13). This is a procedure similar to well-established protocols [282, 365]. While fraught with all the limitations of testing of laboratory-grown, single-species planktonic bacteria, it is a starting point for assessment and comparisons of materials' antibacterial properties.

Bacteria in biofilms-Direct Contact Test (DCT)

Membrane filters (MF-Millipore™ Membrane Filter, 0.45 µm pore, Merck, Darmstadt, Germany) were cut into circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 µl of each bacterial inoculum OD_{600} 1.0 was applied upon the outer surface of the membranes. The agar plates were incubated at 37^{o}C in a 5% CO_2-supplemented atmosphere for 48 hours, and monospecies biofilms were established (Figure 3.4).

For investigating the antibacterial properties against *E. faecalis* in biofilms, we used a 48 hours-grown biofilm model using mixed cellulose esters (MCE) membrane filters [222]. In

previous studies, *E. faecalis* biofilms were grown on biological substrates such as bovine dentine or human dentine [351, 366]. Nevertheless, the tested sealers may firmly adhere on dentine leading to partial retrieval of bacteria or possible carryover effect [282].

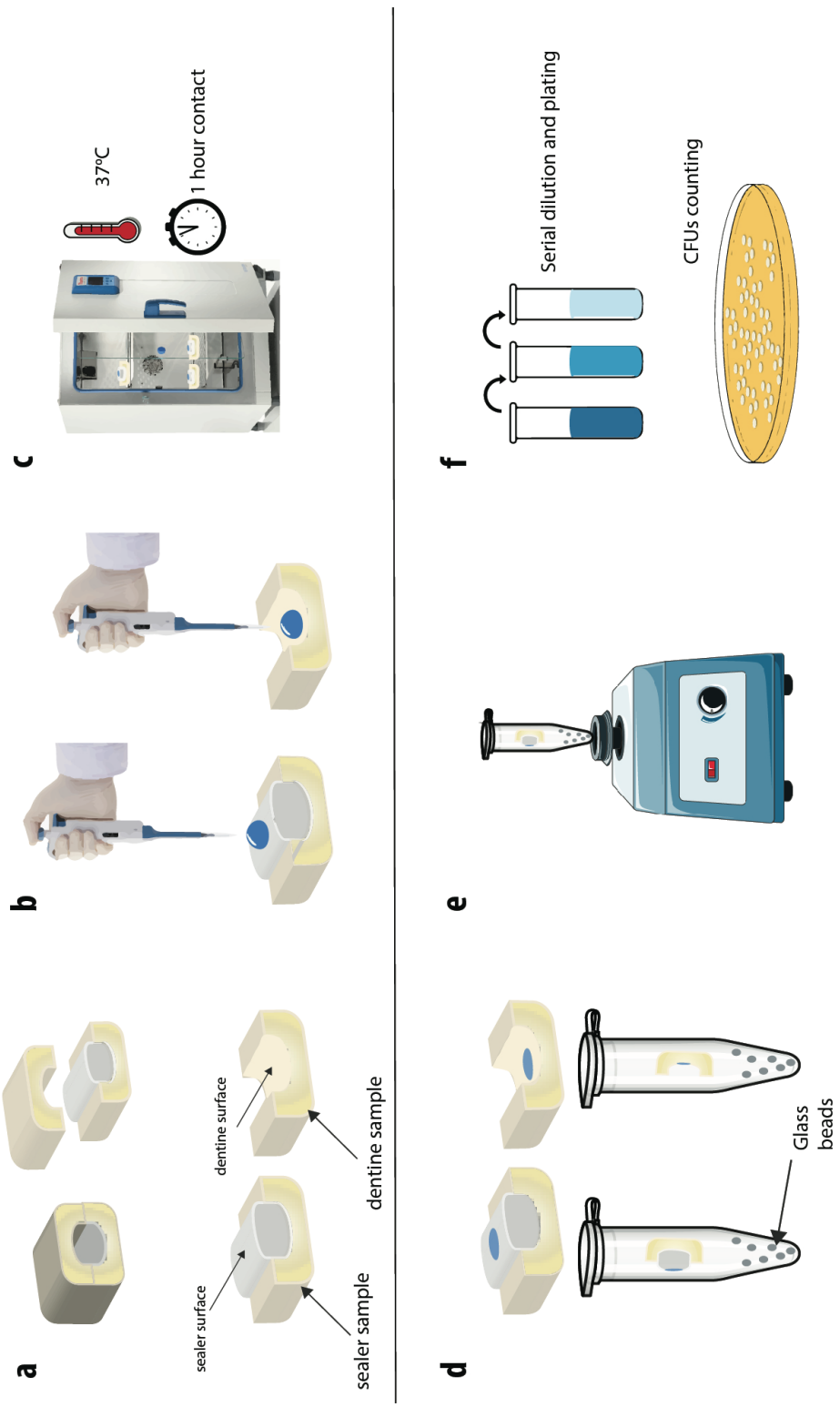

Figure 3.13: Schematic representation of planktonic assay. An amount of 5 µl *E. faecalis* bacterial suspension was carefully placed upon the dentine (dentine samples) and the sealer surface (dentine-sealer sample), or only upon the dentine surface in irrigation groups without sealer and control group (b). The specimens were incubated at 37°C for 1 hour, while complete evaporation of the suspension's liquid was inspected (c). The sealer samples and their adjacent dentine samples were separately transferred in vials containing 500 µl PBS and were vigorously vibrated with glass beads (d and e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO_2 supplemented atmosphere (f).

In our study, the SEM examination showed substantial separation of the sealers from the dentine. In addition, the high hydrophilicity of MCE membrane filters enabled an easy separation of the filter with the biofilm from the sealers, thus minimising biofilm disruption.

The use of a mono-species biofilm model may limit extrapolation of results to clinical applications. But although laboratory models invariably represent a simplification of the clinical reality of the infected root canal, they remain valuable tools for preliminary assessment of endodontic materials and treatment strategies. According to a recent systematic review on root canal biofilm model systems [368], in the vast majority of the studies (86%), the biofilms were composed of only one species, with *E. faecalis* being the most common one (92% of the monospecies biofilms studies). It is an ongoing debate whether and to what extent researchers should try to mimic the complex *in vivo* situation. Mono-species biofilm model systems provide simplicity, and they can be standardised and controlled. Their set-up is easy, reproducibility is good, and they allow high experimental throughput. Our model uses split human teeth to assess the antimicrobial effects of endodontic irrigants on the canal wall dentine and the sealers separately. Emphasis is also put on the applicability and reproducibility of our model and, we therefore favoured the use of a previously established monospecies biofilm model over the complexity of a multispecies biofilm model.

3.5 Statistical methods

The statistical methods applied in this thesis respected the “nature” of the data. Before each statistical analysis, the data were assessed for normality with the Shapiro-Wilk test and homogeneity of variance with Levene's test. The assumptions for each statistical test employed were checked.

In paper I, the antibacterial assays were analysed using the nonparametric Kruskal–Wallis test and Dunn’s post-hoc method due to the 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 ($p < 0.05$).

In paper II, the statistical analysis was performed using the nonparametric Kruskal–Wallis test and Dunn’s post-hoc method due to absence of normal distribution ($p<0.05$). In the case of comparing two groups, non-parametric Mann Whitney U test was performed ($p<0.05$). Moreover, multiple linear regression tests were performed to assess the effect of irrigation (NaOCl, CHX), type of sealer (AH Plus, BioRoot RCS, PCS), substrate (dentine or sealer), ageing period (1-, 7-, 28 days), and dentine pretreatment with EDTA on the bacterial survival both for planktonic bacteria and bacteria in biofilms, using two sets of regressions (one for planktonic bacteria and the other for bacteria in biofilms). All regression analyses were tested for linearity, homoscedasticity, and multicollinearity assumptions, checking for unusual points

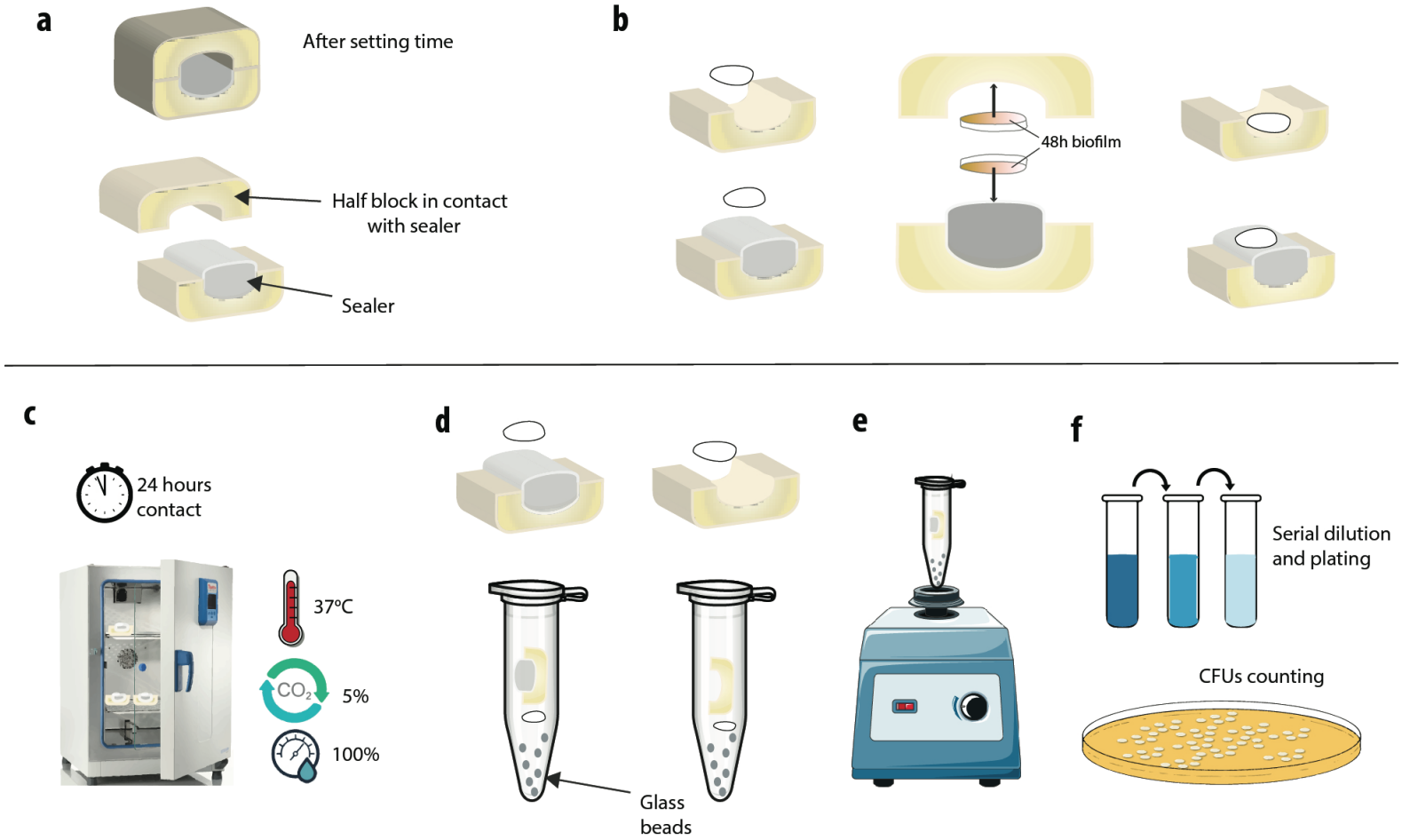

Figure 3.14: Schematic representation of biofilm assay. The filter membranes were positioned upon the dentine and sealers with the established biofilms facing their surfaces (b). The specimens were wrapped with Parafilm M to secure the membrane filters upon the surfaces and placed at 37°C in a 5% CO_2 supplemented atmosphere for 24 hours (c). After 24 hours, each membrane with its corresponding sealer-dentine or sealer block was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads (c and d). After serial dilutions in PBS, colony forming units (CFU) were counted after incubation at 37°C in a 5% CO_2 supplemented atmosphere (f).

(significant outliers, high leverage points or highly influential points) and normality of residuals. All the tested assumptions were met for all the regression analyses performed. In paper III, 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 the 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 the absence of normal distribution of data ($p < 0.05$).

Chapter 4

Discussion of findings

4.1. General

The work presented in this thesis sought to investigate the interactions between irrigation solutions, more specifically CHX, with endodontic sealers. This discussion aims to answer whether the hypothesis hold true that irrigation with CHX would modify the different properties of endodontic sealers. A complete description of results and a detailed discussion of those, are presented in the original papers and manuscripts later in the thesis.

The overall objective of this thesis was to assess the effect of interactions between three endodontic sealers and 2% chlorhexidine dicuglonate (CHX) as an irrigation solution both *in vitro* and *ex vivo*. The primary focus is on the microbiological aspects of these interactions. The secondary aim was to explore the impact of CHX on cytotoxicity, physicomechanical and chemical properties of the tested materials. Both sealer surfaces and leachates were tested, and a tooth model was developed and used to assess the effect of irrigation on the antimicrobial properties of the sealer-dentine interface.

The null hypothesis that CHX would yield non-significant results in any of the antimicrobial, cytotoxicity, physicomechanical and chemical properties of tested endodontic sealers was not supported. CHX was shown to improve the antimicrobial properties of endodontic sealers; it reduced cell viability of leachates; and it affected the physicochemical properties to various extents. Long-term contact with CHX during setting of the sealers lead to higher antimicrobial activity than short-term contact for 1-minute. In addition, contact with CHX improved the antimicrobial properties of sealer leachates. While it has been shown that CHX is an efficient antimicrobial agent [180, 270, 403, 404], findings for cytotoxicity vary [270, 405]. A recent publication evaluating the cytotoxicity of AH Plus, MTA Fillapex (hydraulic calcium silicate -based cement) and PCS with incorporated CHX nanoparticles demonstrated increased cytotoxicity of CHX-modified sealers [270].

In the split-tooth model, NaOCl and CHX affected the antimicrobial properties of dentine and sealer surfaces to various extents. Overall, CHX improved the antibacterial activity of dentine surfaces as well as of the adjoining sealer, and the two irrigation protocols tested differed in antimicrobial efficacy. These results point to a differential effect of irrigation dependent on the mode of application and on the sealer used, and highlight the need for a matched irrigation to root canal filling strategy for root canal therapy, given the diverse chemistry of the means used during root canal irrigation and filling [348]. The customisation of the techniques used in root canal treatment may enhance the therapeutic result of endodontic treatments.

The study design necessitated that the materials be prepared in various setups specific for testing of antimicrobial, physicomechanical, and chemical properties. The objective was to apply the least possible CHX on sealer surfaces in an attempt to mimic the clinical situation. Whereas the basic interaction of irrigating solution with sealer was analyzed in simple tests of leachates from incubations of sealer specimens in irrigation fluid (Papers I and II), a closer-to-clinical approach was attempted with the split-tooth model in Paper II.

The antimicrobial properties of leachates have been mainly tested for pulp capping materials or root-end filling materials [356, 406]. The antimicrobial effects of endodontic sealers' leachates (liquid constituents) are investigated herein for the first time. In addition, few studies have investigated the effects of irrigation on the cytotoxicity of sealers, but some have assessed the leaching of sealers and characterized their leachates [271, 308, 311, 401]. The present thesis tested sealer surfaces for antimicrobial properties against planktonic bacteria and bacteria in biofilms. Data on the antimicrobial properties of sealers against established biofilms are scarce, even though various biofilm models have assessed the effectiveness of endodontic irrigants [344, 345]. Root canal sealers are placed in direct contact with dentinal walls, which may host live bacteria both in planktonic suspensions and in biofilm forms [28, 30]. The presence of residual bacteria can occur even in the main canal, in untouched areas after chemo-mechanical preparation, such as apical ramifications, lateral canals, and isthmuses [28, 358-360]. The findings in our studies of an enhanced antibacterial activity of sealers set onto CHX-exposed dentine may point to a possible supportive effect of the CHX-rinse in disinfecting residual microbes in inaccessible areas.

4.2 Sealer surfaces and leachates

4.2.1 AH Plus

AH Plus, an epoxy resin–based cement, is frequently used as a benchmark for comparisons. Efforts have been made to enhance the antimicrobial properties of the sealer by incorporating antimicrobial agents such as quaternary ammonium, silver nanoparticles, benzalkonium

chloride and CHX [285-288]. We found that most of the physicomechanical properties of AH Plus were modified after contact with CHX. Setting time of AH Plus alone was consistent with manufacturers' guidelines and previous publications [407, 408]. The setting of the sealer in contact with CHX and water was slowed down, seen as an observed reduction in microhardness values. These findings are informative about monomer conversion: The interaction with CHX may interfere with the setting mechanism (delay polymerisation) and plastify the sealer consistency. Interactions between the positive charge of the CHX molecule and the free carboxyl groups in the polymer matrix have been previously described [409, 410]. Although a hydrophobic sealer, AH Plus is also sensitive to moisture from potential contaminants [386]. This was shown in the wettability test, where exposure to CHX and water rendered AH Plus more hydrophilic in a time-dependent manner. These effects in properties could be attributed both to the additional water of CHX aqueous solution and the possible chemical interactivity between CHX and the sealer [386]. However, our FT-IR and XRD analysis showed no evident changes in the chemistry of AH Plus except for an extra calcium carbonate phase under CHX irrigation. These findings are consistent with those of Ruiz-Linares et al., who reported a mean of 577.70 minutes setting time for AH Plus when 2% CHX was incorporated into the sealer, albeit a longer setting was observed in unmodified AH Plus [411]. A recent study assessing the role of residual irrigation solutions and intracanal medications on the rheological properties of sealers showed prolonged setting time for AH Plus in contact with CHX in alignment with our study [386]. The differences between the studies above may be due to the differences in protocols and methods for assessing the samples.

Set specimens of AH Plus had no inhibitory effect on the microbes tested, either planktonic or in biofilms. Our findings corroborate with earlier literature, which indicates that AH Plus loses its antimicrobial efficacy after the setting process [281]. An increase in antibacterial activity was not observed when saline was used, as previously shown in another study using water upon sealers [282], 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; and it is well established that biofilms are more resistant compared to their planktonic counterparts [412]. Exposure to CHX conferred antimicrobial properties to AH Plus, confirming similar findings of Bailón-Sánchez *et al*., who investigated the antimicrobial properties of AH Plus modified with CHX [288]. In biofilm assays against AH Plus surfaces, only long-term exposure improved the effectiveness of the sealer, indicating a time-dependent action against the more resistant biofilms compared to their planktonic counterparts [412].

The amine groups in CHX have the potential to interact with the free carboxyl groups in the polymer matrix of resin, serving as a cross-linking agent [410]. This binding property may favour CHX's substantivity to resin and contribute to extra antimicrobial efficacy [413]. 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 effectiveness only as unset [281, 282]. An explanation for this is AH Plus' physical properties and chemical stability [278, 279]. Any compounds that potentially have an antimicrobial effect may be entrapped in the resinous matrix [414].

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 [222] or incorporation of CHX [288]. 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 [270, 353, 354, 415-418]. AH Plus contains epoxy resin that is cytotoxic [419], and this may explain the pronounced cytotoxic effect of the sealer, particularly as freshly mixed [416].

4.2.2 BioRoot RCS

Hydraulic calcium silicate-based sealers interact with dentin [420]. Therefore, the final irrigating solution's residuals may affect the sealer integrity and antimicrobial properties. Hydraulic calcium silicate-based cements, such as BioRoot RCS, have highly hydrophilic surfaces [222], leading to increased water adsorption and porosity. BioRoot RCS combine both good antimicrobial properties and high biocompatibility [271]. No efforts to modify BioRoot RCS with CHX have been reported yet, while earlier literature has been focused on the effects of CHX on MTA and other root repair materials [404, 421-426]. A few studies have assessed the interactions between calcium-phosphate cements and CHX irrigation in the bond-strength to dentine with inconclusive results [426-429]. One study on the effect of 2% CHX irrigation on BioRoot RCS in terms of push-out bond strength showed no differences with other irrigants (3% NaOCl, 20% citric acid or 0.9% NaCl) but 17% EDTA.

In our study, BioRoot RCS more than AH Plus and PCS was affected by CHX in relation to physicomechanical properties. BioRoot RCS sets faster than the other two sealers tested [301-304]. Nevertheless, short- and long-term exposure to CHX almost doubled and tripled the setting time respectively. Moreover, exposure to water alone also prolonged the setting, suggesting that it is the aquous environment rather than the CHX which causes the delay in setting. Adding 2% CHX to MTA prevented setting of the material for long time periods [421, 422]. The water-to-powder ratio has been shown to be crucial for the rheological properties of hydraulic cements [430]. In this respect, the differences further reported in the microhardness assay are in accordance with the setting behaviour of BioRoot RCS under CHX exposure.

Hydraulic calcium silicate-based cements, such as BioRoot RCS, present increased water adsorption due to their surfaces' high hydrophilicity. Moreover, its hydraulic nature and the formation of calcium hydroxide render the sealer susceptible to environmental conditions [311]. The microscopic images further confirmed these differences in physical properties as BioRoot RCS appeared porous with capillary voids. 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 [304]. In addition, a study on the setting of a premixed calcium phosphate silicate–based sealer (EndoSequence BC Sealer) documented a reduction in microhardness when water was included in the sealer [418]. BioRoot RCS in root canals has been found to present more voids than AH Plus, a result that is reflected in the higher values in surface roughness reported for the sealer in our study [312]. 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 the growth of residual bacteria [398]. Microleakage models using glucose as a 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 [399, 400].

Exposure to CHX, which constitutes a positively charged hydrophobic molecule, significantly decreased the wettability of BioRoot RCS in a time-dependent manner. Interestingly, this tendency was not shown for pure water, as no drops could be formed for angle measurements. In the tooth model, BioRoot RCS upon dentine presented the calcium phosphate phase, after CHX as well as saline irrigation. The peaks of calcium hydroxide were lower in the tooth model than in the control (cylinder specimens of the sealer, paper I), indicating a reaction to form calcium phosphate. These findings suggest a role for the smear layer in calcium phosphate formation and that CHX may not interfere in this process. On the contrary, a previous study has shown that a final irrigation with EDTA did not promote the formation of calcium phosphate [337], even though a beta-like calcium phosphate has been earlier proven to form when the sealer was immersed in simulated tissue fluids [301]. XRD and FT-IR analyses demonstrated that the chemistry of the sealer remained unchanged under CHX and saline irrigation.

BioRoot RCS surfaces exerted better antimicrobial properties after exposure to CHX. The proposed antimicrobial mechanism of calcium-silicate cements is predominantly related to high alkalinity: calcium hydroxide, principally formed out of the hydration process, releases calcium ions (Ca^{+2}) and hydroxyl ions (OH^{-}) in water. BioRoot RCS leachates also eliminated all the planktonic bacteria for all setting times, and it showed antibacterial activity up to 7 days against *E.faecalis* and *S.mutans* biofilms. The high alkalinisation effect of BioRoot RCS was also reported in this study, a finding that is consistent with previous data [311]. CHX contact did not compromise the antibacterial properties of BioRoot RCS leachates 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. The enhanced antimicrobial behaviour of hydraulic cements after modification or contact with CHX may be further explained by the synergistic release of calcium and hydroxyl ions and CHX, given their high solubility [270]. There are few studies assessing the antimicrobial effectiveness of BioRoot RCS only against *E. faecalis* with contradictory results, partially explained by differences in methodology [337, 431, 432]. Moliz et Camilleri, using an agar diffusion test, showed high antimicrobial efficacy for BioRoot RCS and especially after a final irrigation with EDTA in an *in vivo* simulated tooth model [337]. In a recent publication assessing the antimicrobial efficacy of sealers against *E. faecalis* biofilms upon dentine using CSLM, BioRoot RCS presented fluctuations over time [431]. One study used a modified DCT in conjunction with ADT, concluding in moderate antimicrobial properties for BioRoot RCS [432]. Earlier literature has shown better antimicrobial properties for MTA mixed with CHX [404, 423-425, 433, 434]. Two studies have investigated the antimicrobial properties of Biodentine, a calcium-silicate cement with similar chemistry to BioRoot RCS, combined with CHX, showing improved efficacy compared to unmodified cement [403, 404]. Due to differences in methodology and tested bacteria between previous studies and the current, no direct comparisons can be performed, and the extrapolation of conclusions is challenging.

Furthermore, BioRoot RCS leachate exerted the lowest cytotoxicity among the sealers tested. BioRoot RCS is a bioactive calcium silicate sealer showing favourable results on human periodontal ligament cells [353, 435, 436]. Moreover, the low cytotoxicity of hydraulic calcium silicate cements may also be associated with pronounced calcium ion release and the high alkalinisation potential of these materials [406]. Previous studies on sealer cytotoxicity have also shown less cytotoxicity for BioRoot RCS than AH Plus and PCS [353, 354]. Interestingly, BioRoot RCS with CHX contact was the only sealer with lower cytotoxicity compared to CHX positive control for all setting times (Paper III).

4.2.3 Pulp Canal Sealer

Pulp canal sealer is a traditional eugenol-containing sealer that has been in clinical use for decades and that possesses good antimicrobial properties but controversial biocompatibility [322]. In the past, zinc-oxide eugenol cements have been mixed with additives in an attempt to tailor their properties and enhance clinical performance [323-327]. Back in 1984, Nambu prepared a prototype ZOE-based root canal sealer with the addition of 1% CHX, achieving higher antimicrobial performance than unmodified ZOE cements [328]. Regarding interactions between CHX irrigation and ZOE based sealers, two studies have investigated the effect of CHX on the short- and long- term apical seal of Roth's 811 with no adverse effects [5, 437].

In the present study, PCS alone exhibited a mean setting time of 221 minutes, relatively close to manufacturer's indication (2 hours), taking into account the wide fluctuations in setting of the sealer previously reported in the literature [267, 308, 438]. Our findings indicate 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 has been proven to shorten the setting time of ZOE cements given that CHX in aqueous presentation was applied upon the sealers [439].

PCS, a highly hydrophobic material [304], became more hydrophilic after CHX and water contact in the same pattern as AH Plus. Presumably, the abundant moisture from the aqueous CHX increased the sealers' hydrophilicity in a time-dependent manner. As a hydrophobic material, PCS does not favour water adsorption and consequently exhibits low porosity [304]. PCS has displayed pronounced shrinkage when stored at 100% humidity [267]. Similarly, a zinc oxide-eugenol impression material presented reduction in dimensions after disinfection with aqueous CHX solutions [440]. Thus, exposure to CHX may have led to shrinkage of PCS which in turn resulted in pore and gaps reduction within the sealer bulk and, finally, in lower surface roughness values.

PCS sealer exhibits low values of compressive strength [441]. We also found low values for microhardness for the sealer in our study, values that were further reduced by CHX exposure. This might be partially explained by the two extra phases, namely sodium silver chloride and silver aluminium, that PCS demonstrated under CHX irrigation, which may negatively affect the mechanical properties of the sealer.

In general, PCS alone and in contact with CHX exerted the strongest antimicrobial properties among the tested sealers. Our results for PCS alone align with the literature, as zinc oxide and eugenol sealers have been reported to be efficient towards microorganisms, notably during and before setting [322, 327, 351, 417, 442, 443]. Free-leaking eugenol is the first contributing factor to the pronounced antimicrobial efficacy of PCS, which is also consistent with the high antimicrobial properties of PCS against both planktonic bacteria and bacteria in biofilms [334, 335]. Release of eugenol was also indicated in our study, given the negative water uptake values and the yellowish colour of PCS leachates. Few studies have endeavoured to incorporate CHX to ZOE-based cements, achieving better antimicrobial properties [328, 444, 445]. Nambu attempted to incorporate CHX to a ZOE sealer and described a possible mechanism of CHX gradual release; the author assumed that free eugenol remains in the set ZOE sealer, and consequently CHX dispersed in the free eugenol is released from the set sealer [328]. This might give an explanation of the enhanced antimicrobial properties of PCS in contact with CHX in our study. Moreover, the extra silver chloride phase identified under irrigation with CHX is well documented for its antimicrobial properties and may have further contributed to the advanced antimicrobial efficacy of PCS [446].

PCS was the material to be affected most 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 a dry surface texture with significant cracks in the bulk of the material, an indication of extensive shrinkage. Contact with CHX reduced the number of cracks on the surfaces, while more voids were evident. Release of eugenol may

also be associated with the presence of microcracks and shrinkage. Pronounced shrinkage for PCS has been observed when stored at 100% humidity [267], as well as the dimensions of a zinc oxide-eugenol impression material were reduced after disinfection with aqueous CHX solutions [440]. Additionally, PCS is a hydrophobic material [222] and thus does not promote water adsorption and consequently exhibits low porosity [304], findings that corroborate with the present study. Furthermore, we found evidence for the presence of silver and zinc oxide in PCS, which also contribute to the antimicrobial properties of ZOE-based cements [447, 448]. FT-IR analysis indicated that the resin in the resin block altered the PCS chemistry. The interference of eugenol with the setting of resins is well known [449, 450]. The use of a resin block is thus not indicated for testing PCS, but it was useful to show the changes attributed to the contact with dentine.

PCS leachates from freshly mixed specimens in contact with CHX showed high cytotoxicity, which corroborates previous scientific data [270, 353]. Eugenol release has commonly been associated with ZOE cytotoxicity [336].

4.3 Findings particular to the split tooth model

A split tooth model was developed to examine the residual antimicrobial effect of two irrigants and two clinical irrigation protocols at the level of the sealer-dentine interface. More explicitly, both the dentine and the sealer surfaces, which had been in contact with each other, were assessed for their antibacterial properties.

The split tooth model was first verified for its applicability by means of SEM and elemental analysis to secure complete separation of the sealer bulk from dentine. The SEM examination showed no cohesive failure, which would have resulted in dentine surfaces being covered with sealer after separation. The model was therefore considered suitable for investigating surface characteristics after separation.

Previous studies have mainly used a dentine infection model *(ex vivo* model for infection of dentinal tubuli) to assess the effectiveness of either endodontic irrigants [344, 345, 451] or root canal sealers inside the dentinal tubuli [346]. Our study is the first to measure the combined antibacterial effect of irrigation and endodontic sealers on dentine walls and sealer surfaces simultaneously.

Most endodontic sealers maintain their antibacterial properties throughout the setting process [282]. Among the tested irrigants, CHX can bind to dentine and be gradually released. This may contribute to prolonged antibacterial properties [159, 217]. In the present study, the incubation time ranged from 1 day to 28 days for assessment of the irrigation's potential long-lasting antibacterial effect on sealers. A short contact time may not be adequate and representative of the total antibacterial capacity of materials, therefore, we tested the antibacterial properties against established biofilms for 24 hours contact time. The sealers

were applied in bulk without a gutta-percha core, which was necessary to enable gentle and complete sealer detachment from the dentine. In order to assess the isolated effect of 1% NaOCl and 2% CHX on antibacterial properties, the smear layer was removed with the use of 17% EDTA and the root blocks were rinsed in between with saline solution to avoid any additional interactions between EDTA and NaOCl-CHX [174]. As clinical procedures most often entail the use of several irrigation liquids, two relevant irrigation protocols were also tested: 1% NaOCl + 17% EDTA and 1% NaOCl + 17% EDTA + 2% CHX. Only treatment with CHX eliminated the planktonic bacteria on dentine surfaces for all incubation times up to 28 days. This result corroborates earlier literature on CHX's ability to possess long-lasting antibacterial properties due to substantivity [217, 218, 452]. In the present model, 1% NaOCl had inferior antibacterial properties to 2% CHX, which can be potentially attributed to its low concentration; *in vitro* studies indicate that a higher percentage of NaOCl could result in increased antibacterial properties [185, 453]. However, clinical findings suggest no significant differences in the antimicrobial properties of NaOCl in different concentrations (0.5%–5.25%) [454, 455]. Moreover, a recent randomized clinical study reported similar clinical outcomes for high (5%) and low (1%) NaOCl concentrations [456]. Toxicity of NaOCl to periapical tissues as well as its deleterious effect on the integrity of dentine structure and on the collagen matrix is concentration dependent, with higher concentrations being more irritating [198, 348, 457, 458]. Thus, in our study, 1% NaOCl was preferred to higher percentages as low NaOCl concentrations have been shown to combine both antimicrobial properties and low cytotoxicity. Application of CHX managed to reduce significantly the numbers of *E. faecalis* in biofilms only after an 1-day incubation period, confirming that biofilms are more resistant than their planktonic counterparts [412].

AH Plus possesses antibacterial properties mainly during the setting of the material [281, 282]. We also found persistent antibacterial activity of AH Plus unexposed to CHX or NaOCl. However, AH Plus and dentine surfaces exerted antibacterial properties against both *E. faecalis* planktonic bacteria and biofilms when CHX was applied. Exposed to NaOCl AH Plus dentine surface eliminated the planktonic *E. faecalis* after 1 day of incubation, and reduced *E. faecalis* in biofilms after 1- and 7 days incubation, confirming the additive effect of NaOCl and AH Plus shown in an *ex vivo* study [352].

BioRoot RCS sealer surfaces eliminated planktonic *E. faecalis* in all groups and incubation times. The proposed antibacterial mechanism of BioRoot RCS is based on the hydration of tricalcium silicate-based cements [222, 301, 459, 460]. BioRoot RCS was found to be strongly antibacterial against *E. faecalis,* especially after final irrigation with EDTA, in an *ex vivo* intratubular infection tooth model study [337]. Our results corroborated these findings: a final application of EDTA increased the antibacterial properties of BioRoot RCS. Even though EDTA has been found to interact with the tricalcium silicate and reduce or eliminate the formed calcium hydroxide [248, 337], the antibacterial properties of the sealer were not compromised in the present study. This can partially be explained as EDTA chelates calcium from the sealer and the dentine, providing more free calcium and thus increasing the

antibacterial activity [337]. Moreover, the residual effect of CHX enhanced the antibacterial efficacy of BioRoot RCS dentine surfaces. One study found that the antibacterial properties of BioRoot RCS against *E. faecalis* biofilms in dentinal tubules presented fluctuations over time [431]; another concluded that BioRoot RCS had moderate antibacterial properties using a modified DCT [432]. Two recent studies showed strong antimicrobial activity for BioRoot RCS [460, 461]. As mentioned before, variable results for the antibacterial properties of BioRoot RCS seem most likely to be reported due to differences in methodology [337, 431, 432].

PCS exhibited antibacterial properties mainly on sealer surfaces which had been in contact with dentine and high efficacy against *E. faecalis* biofilms. This indicates that PCS may exhibit moderate constant antibacterial properties related to the gradual release of eugenol [334, 335]. Moreover, a new study demonstrated a decrease in *E. faecalis* live bacteria upon PCS surfaces after an initial biofilm formation, which may correlate to zinc release [460]. To the contrary, the antibacterial effect of dentine that was in contact with PCS was weak, especially against biofilms. This could be attributed to the pronounced shrinkage that PCS displays stored at 100% humidity [267], which might lead to loose (non-tight) contact with the dentinal walls and thus compromised antibacterial properties. Moreover, a zinc-oxide eugenol impression material reduced dimensions after disinfection with aqueous CHX and NaOCl solutions [440]. Previous studies on zinc oxide eugenol cements as PCS have demonstrated improved antibacterial activity after mixing with CHX [328, 444].

Chapter 5

Concluding remarks

The present thesis aimed to investigate the potential interactions between endodontic sealers and irrigation (especially 2% CHX). The primary focus was on the microbiological aspects of these interactions evaluating the antimicrobial properties of sealers. The secondary was to further explore the impact of CHX on physicomechanical, chemical and cytotoxicity properties of the tested materials. Also, we developed an *ex vivo* tooth model to assess whether the residual presence of 1% NaOCl or 2% CHX may augment or reduce the antibacterial properties of dentine and endodontic sealers.

- The results of these studies lead to the conclusion that CHX may improve the antimicrobial properties of the sealers. Contact with CHX had an apparent added antimicrobial effect, especially for AH Plus. PCS with and without CHX contact presented the highest antimicrobial efficacy of surfaces among the tested sealers, while BioRoot RCS with and without CHX was the most antimicrobial sealer for leachates. In the *ex-vivo* tooth model, CHX increased the antibacterial activity in relation to sealer and dentine surfaces. Overall, BioRoot RCS and PCS presented extended antimicrobial efficacy, whereas AH Plus was antimicrobial only during setting.
- Contact with CHX increased cytotoxicity for all sealers. Among the tested sealers, BioRoot RCS leachates presented the least cytotoxicity with and without CHX contact.
- The physicomechanical properties of all three sealers were affected in various extents after CHX contact. Surface characterisation showed no changes for AH Plus and BioRoot, while two extra phases were observed for PCS after contact with CHX.
- The alkalinity of sealer leachates did not change after CHX contact. BioRoot RCS presented the highest pH values.
- The split tooth model was first verified for its applicability by means of SEM and elemental analysis to secure complete separation of the sealer bulk from dentine. The SEM examination showed no indications of cohesive failure, which would have resulted in dentine surfaces being covered with sealer after separation. There was complete separation of the sealers from dentine at the sealer-dentine interface (adhesive type of failure), and the chemical analyses of the surfaces similarly indicated

separation of sealers from dentin. The model was therefore considered suitable for investigating surface characteristics after separation.

The present thesis may contribute to a better understanding and knowledge of the potential interactions between endodontic sealers and irrigation solutions. Customisation of the techniques and materials used in endodontic treatments may ensure that root canal fillings maintain their antimicrobial properties over time without compromising their physicochemical performance.

The overall hypothesis that CHX would yield non-significant results in any of the antimicrobial, biocompatibility, physicomechanical and chemical properties of tested endodontic sealers was rejected. CHX is an irrigant with high antimicrobial efficacy, moderate cytotoxicity and affected the physicochemical properties of the sealers to various extents depending on the sealers' chemistry.

Chapter 6

Future perspectives

Endodontics is currently going through one of the most exciting periods of advancement in knowledge within the history of the discipline, especially in terms of the future clinical translation of scientific knowledge. Over the years, many experimental and clinical studies have been carried out to develop and test new endodontic materials with safe biological characteristics and enhanced properties. This thesis conveys new knowledge within these areas and conforms with the requirements for reporting novel research, presenting cause-and-effect relationships for experimental studies and validating new methodologies.

- One of the objectives of this thesis was to establish concrete protocols of *in vitro* biofilm formation that could be widely applied in *in-vitro* studies. This could be done by developing/manufacturing a device for biofilm growth that would be easily monitored and adopted by researchers for their experiments. In this series of experiments, monospecies biofilm models were developed. However, the more realistic clinical scenario is the presence of polymicrobial biofilms in the root canal system. A well-established multispecies biofilm model will give new insight into the antibacterial activity of endodontic materials. In addition, the wide acceptance of this model by the endodontic scientific community is of great importance for future research endeavours; researchers could use the same experimental model to study endodontic biofilms.
- In addition, dentine has been proven to prolong the antimicrobial efficacy of endodontic sealers even after their setting [351]. CHX is also known for its substantivity (ability to bind to dentine), which contributes to the gradual release of its antimicrobial properties [159, 217, 218]. The importance of root canal sealers in the substantivity of CHX should be tested in more detail [218, 452].
- The antimicrobial effectiveness of endodontic substances over time is at the centre of scientific attention, and thus next endeavours should focus on the long-term antimicrobial properties of endodontic materials. 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 [377, 378].

- The use of a mono-species biofilm model is an evident limitation of our study. Irrigants and root canal sealers should also be tested in more complex environments such as multispecies biofilms [352]. Even though simplified laboratory models do not represent the clinical reality of the infected root canal, they constitute valuable tools to preliminary assess the antibacterial effect of irrigation solutions and endodontic materials as they can be standardised and controlled. Their setup is easy and reproducible, allowing high experimental throughput [462]. This study aimed to develop and use a suitable tooth model for testing the antibacterial properties of both endodontic sealers and their adjacent dentinal walls after exposure to CHX and NaOCl. The lack of standardized methods for testing of antimicrobial properties of sealers is a challenge [463]. The use of tooth models to examine the potential interactions between irrigants and endodontic sealers at the sealer-to-dentine interface and dentinal tubules may be of clinical relevance. A standardised tooth model may provide new insights into the antibacterial activity of endodontic materials.
- The clinical scenario should be replicated in a tooth model testing protocols with planktonic bacteria and biofilm formation and assessing the role of dentine, smear layer and sequencing of irrigants in antimicrobial properties. At the same time, sealers' role in irrigants' residual antimicrobial properties should be further assessed [413].
- After the possible interactions in the restrained space of root canals, it is essential that both endodontic irrigants and sealers remain antimicrobial and do not induce cytotoxic effects to the surrounding periapical tissues. Hence, in future, it is crucial to test both the antimicrobial properties and cytotoxic effects of root canal fillings as one entity to make studies like ours more clinically relevant.
- A challenge in endodontics is the individualisation of root canal therapies in regard to the microbiological milieu in each root canal [464]. In this respect, further studies should be performed to investigate the best filling material in relation to the irrigation regime previously used or vice versa. Customisation of the techniques and materials used in endodontic treatments would ensure that root canal fillings as a whole maintain their antimicrobial properties over time without compromising their physicochemical performance. The potential interaction between endodontic irrigants and sealers needs to be further investigated, as there is scant scientific data on the

matter. 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 NaOCl.

- Although many *in vitro* and *ex vivo* studies have demonstrated a wide range of antibacterial efficacy among endodontic materials, clinical studies indicate no significant differences among different endodontic sealers and irrigation solutions regarding clinical outcome [82, 465, 466]. The success of endodontic treatment is multifactorial, with each distinct procedural step playing a significant role and contributing to the overall therapeutic result. Future clinical studies are needed, especially for relatively new endodontic materials, as the calcium silicate-based cements.
- 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 characterization of sealer leachates may thus be of clinical relevance. Moreover, the assessment of leachates of endodontic materials has attracted attention, and the characterization of elution/degraded materials along with cytocompatibility should also be tested *in vitro* [463]. Sealer leachates should be investigated further, including thorough chemical characterization of the eluates. The antibacterial properties of leachates may aid in the eradication of residual planktonic bacteria or bacteria in biofilms in untouched areas extraradicularly in periapical tissues [28, 351, 357-362]. At the same time, the leachable compounds should ideally not induce cytotoxic effects on the periapical tissues as this may retard the healing process and thus jeopardise the clinical success of root canal therapies [264, 363]. Hence, besides surfaces of endodontic sealers, testing the properties of their leachates may be of clinical relevance and could be directly correlated to any potential antimicrobial or cytotoxic behaviour without the interference of the surface characteristics of materials [467-469].
- Further studies assessing the combined antibacterial properties of various endodontic filling materials and irrigants both at the sealer-to-dentine interface and in the dentinal tubules should be performed using multispecies biofilms in *ex vivo* tooth models. Moreover, the use of human cells or clinical bacterial isolates may increase our knowledge of therapeutics for endodontic pathosis.

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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 *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.

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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% CO_2 supplemented atmosphere. The bacteria were suspended in Phosphate Buffered Saline to an optical density at 600 nanometers (OD_{600}) of 1.0, corresponding to approximately 2×10^8 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 (≈ 0.53 μl 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% CO_2 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 OD_{600} 1.0 was applied upon the outer surface of membranes. The agar plates were incubated at 37 °C in a 5% CO_2 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) (Fig. 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 60× water lens, 0.5 μm step size, and a format of 512 × 512 pixels corresponding to an area of 88 × 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 (≈ 0.79 μl CHX/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% CO_2 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% CO_2 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 (≈0.32 μl CHX or water/mm^2). 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

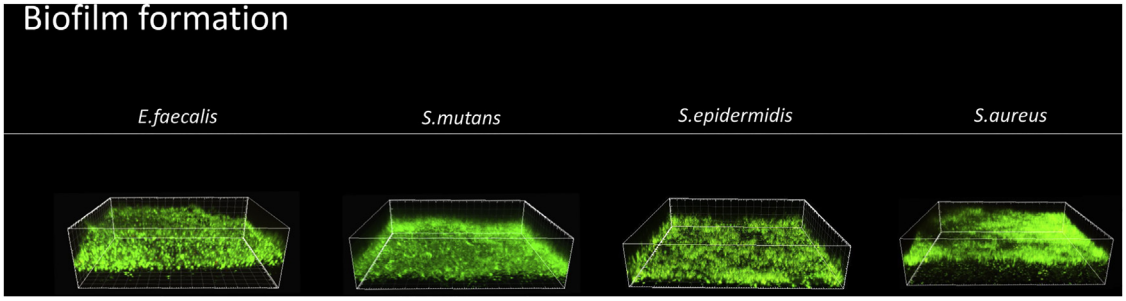

Fig. 1 – Indicative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-hrs monospecies biofilms grown on membrane filters. The scanning was performed from the top of the biofilm to the membrane surface using a 60× water lens, 0.5 μm step size,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

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-μL 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, Rødevre, 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 μm. 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 u 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 non-parametric 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 proper-

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).

Planktonic bacteria	AH Plus		BioRoot RCS		PCS		Controls
	no saline	saline	no saline	saline	no saline	saline	
E. faecalis							
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)*	
S. mutans			*				
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)*	
S. epidermidis					*		
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)*	
S. aureus					*		
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 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. 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).

Biofilms	AH Plus	BioRoot RCS	PCS	Controls
E. faecalis				
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)*	
S. mutans				
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)*	
S. epidermidis				
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)*	
S. aureus				
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 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).

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]

ties (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 Fig. 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 (Fig. 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 (Fig. 3A). BioRoot RCS had two additional peaks, for magnesium and aluminium, to the ones identified in saline group, namely silicon, calcium, chlorine and zirconium (Fig. 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 (Fig. 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 CHX irrigation in both tooth and resin model effected the chemistry of the PCS as indicated by changes in the 2000-2500 cm^{-1} region (Fig. 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} (Fig. 4A).

XRD analysis showed differences in the amounts of the phases and the crystallinity of the sealers (Fig. 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°2θ 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°2θ 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.

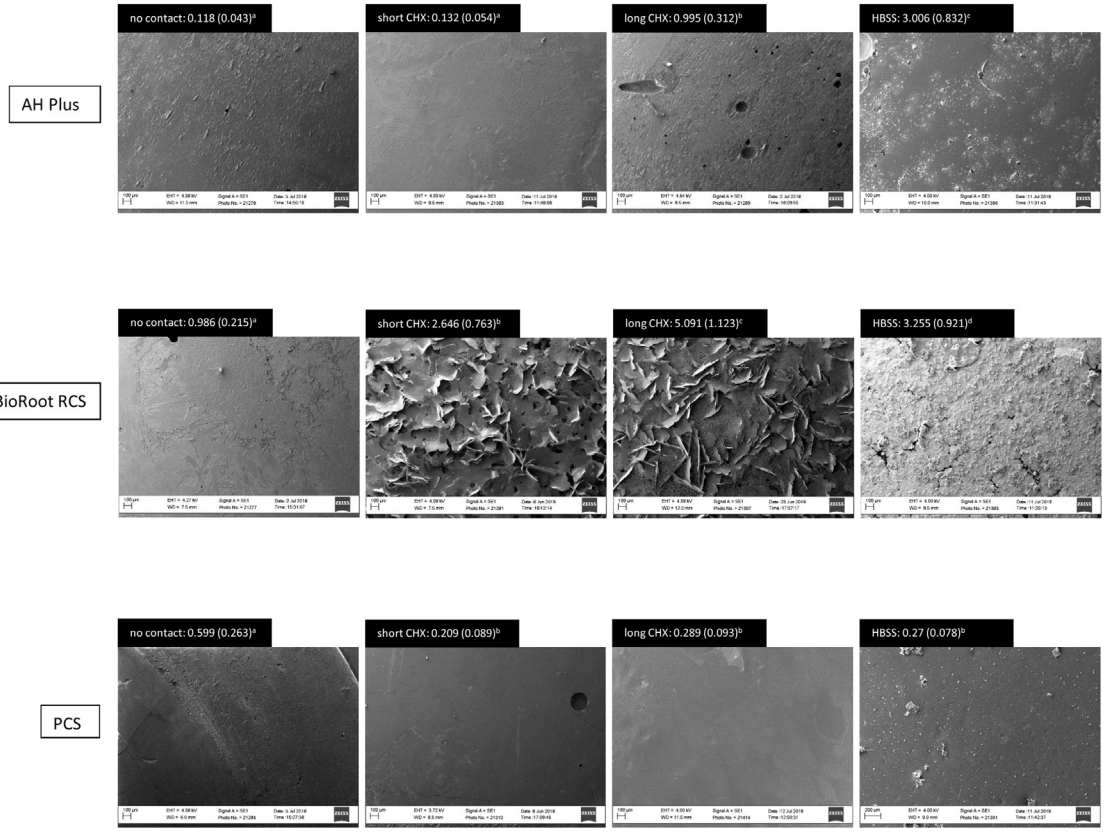

Fig. 2 – Representative secondary electron scanning micrographs of the tested sealers after contact with CHX and HBSS (magnification 100×). Mean and standard deviation of surface roughness Ra. Read horizontally, the same superscript letter shows no statistically significant differences within the same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

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'

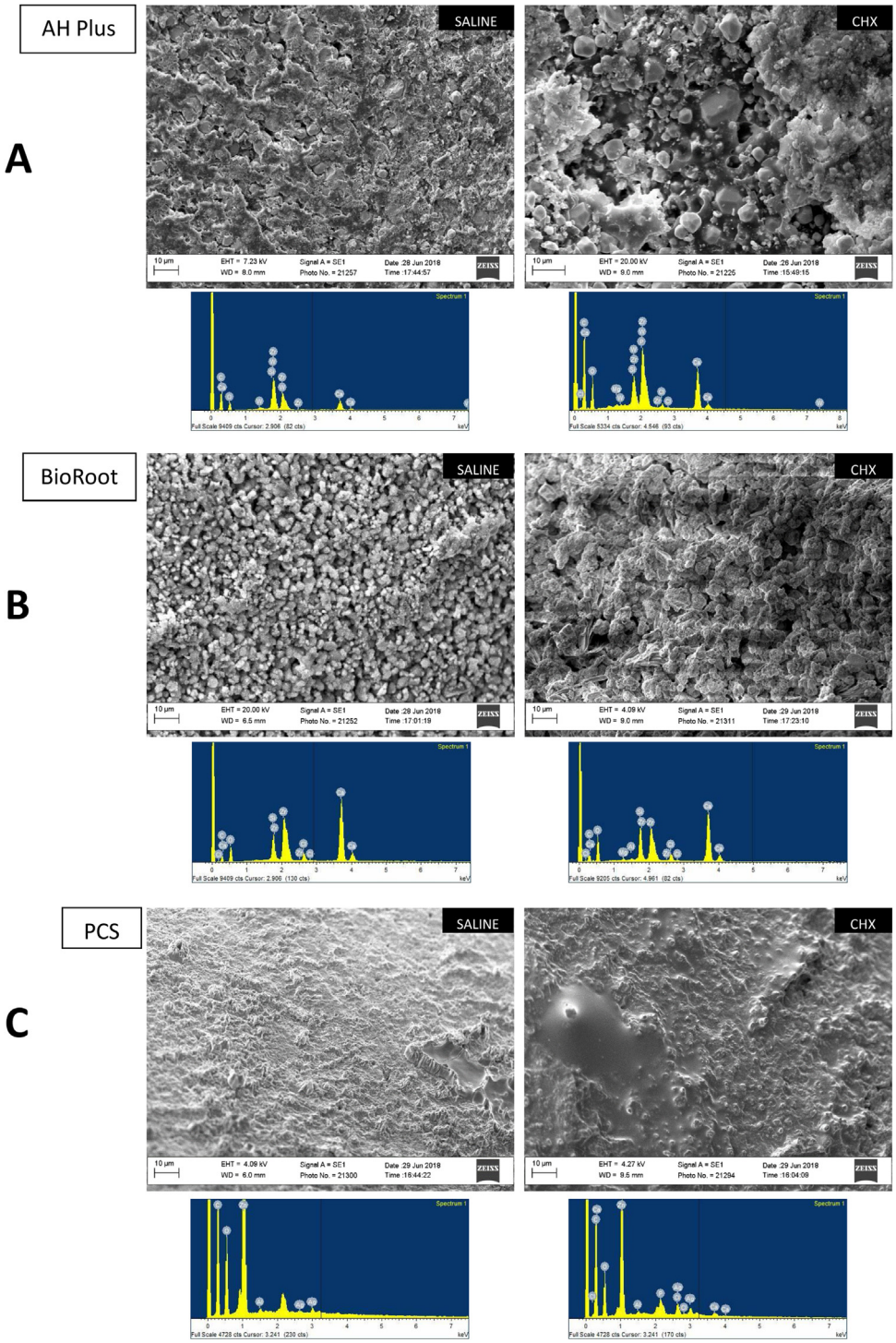

Fig. 3 – High magnification scanning electron micrographs (2000×) of tested sealers retrieved from the split tooth model after saline or CHX last irrigation. Elemental analysis of sealer surfaces and their spectra. CHX irrigation affected the surface microstructure of AH Plus (A), BioRoot RCS (B) and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

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

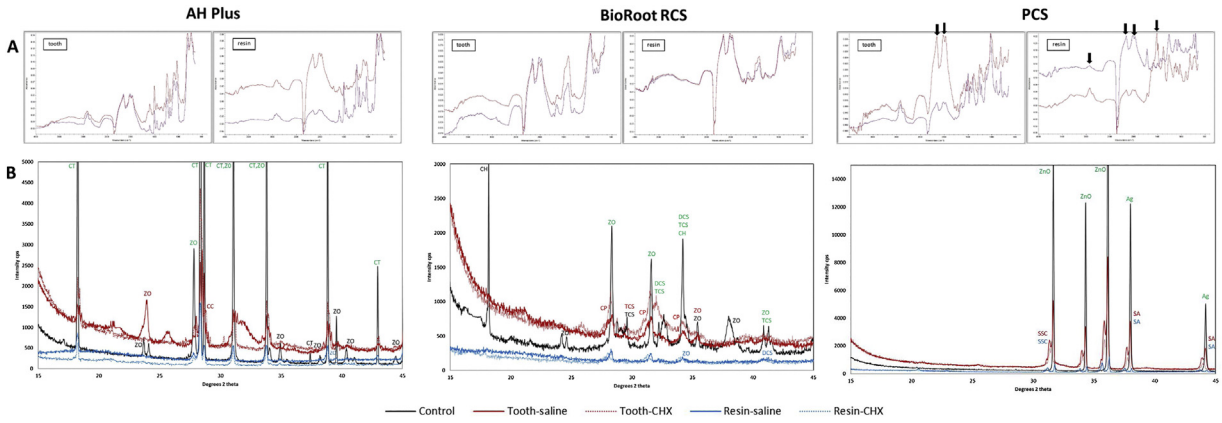

Fig. 4 – (A) Fourier transform infrared spectroscopic scans of sealers placed in a tooth model and resin blocks after final irrigation with saline (blue) and chlorhexidine (red). The black arrows denote the regions (cm^{-1}) where changes in scan patterns are observed (B) X-ray diffraction analysis: 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.

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.

5. 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.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dental.2020.11.011.

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ORIGINAL ARTICLE

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

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Abstract

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

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

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

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

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KEYWORDS

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Endodontic sealers and irrigating solutions

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

Tooth model

Preparation of root blocks

Extracted human teeth were collected from a bio-bank ("2013/413 NIOM tannbank") approved by the Regional Committees for Medical and Health Research Ethics (REC, application number 28748), Norway. All teeth were decoronated and their roots were horizontally sectioned at the apical parts, at a level to form root blocks with a standardized length of 7 mm, using a precision cutting machine (Buehler 11-1280-160 Isomet Low Speed Saw, Buehler; Figure 1a,b). The roots were instrumented with ProTaper rotary files (Dentsply Maillefer) up to size F4, and further enlarged with fibre post drill (3 M Relyx Fiber Post Drill No 3, 3 M, St. Paul, MN, USA; Figure 1c). Oval-shape root canals were prepared measuring approximately 4 mm at the largest diameter (semi-major axis). Irrigation with 2 ml of 1% NaOCl was followed between the changes of the rotary files and a last rinse with 0.9% saline using a 27 gauge Monoject 3cc Endodontic Syringe (CardinalHealth). The root blocks were further segmented (dichotomized) vertically with the use of the diamond saw and the two segments were repositioned and held tightly together by wrapping them up with the use of Parafilm M (Bemis; Figure 1d,e).

Irrigation regimes and obturation

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

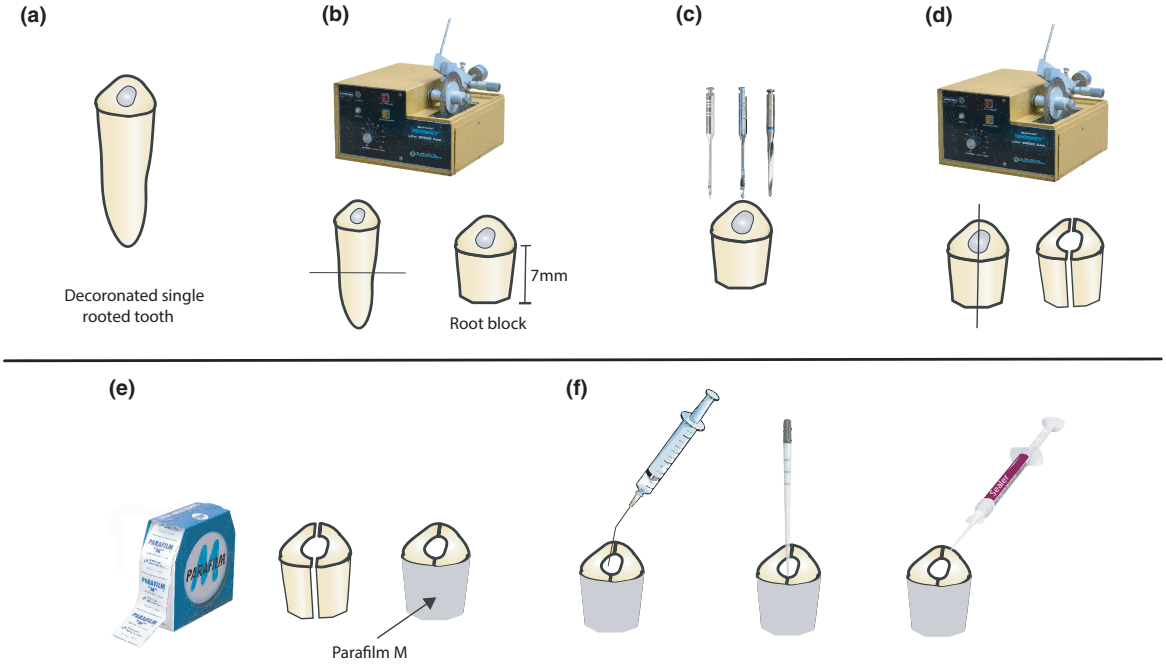


FIGURE 1 Schematic representation of teeth preparation. All teeth were decoronated (a) and their roots were horizontally sectioned at the apical parts, at a level to form root blocks with a standardized length of 7 mm, using a precision cutting machine (b). The root canals were instrumented with rotary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

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

The residual effect of 1% NaOCl (G1) and 2% CHX (G2) as well as the antibacterial effect of two irrigation protocols, Protocol 1 (P1: 1% NaOCl + 17% EDTA) and Protocol 2 (P2: 1% NaOCl + 17% EDTA + 2% CHX), were tested upon dentine which had been in contact with sealers as well as the sealers facing the subjacent dentine (Figure 2a). The antibacterial properties of sealers without any irrigant applications were investigated (G3). In addition, the antibacterial effect of the irrigation solutions and protocols were evaluated on dentine without sealer application. All irrigants were applied from the top of the root blocks formed after tight repositioning of the two segments (Figure 1d,e). In G1, G2 and G3, dentine was pre-treated with 17% EDTA for 5 min (removal of smear layer), rinsed with 2 ml 0.9% saline and dried with paper points (size 40, Reciproc blue, VDW). In P1 and P2, 17% EDTA was not used as dentine pre-treatment but NaOCl was the first irrigant.

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

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

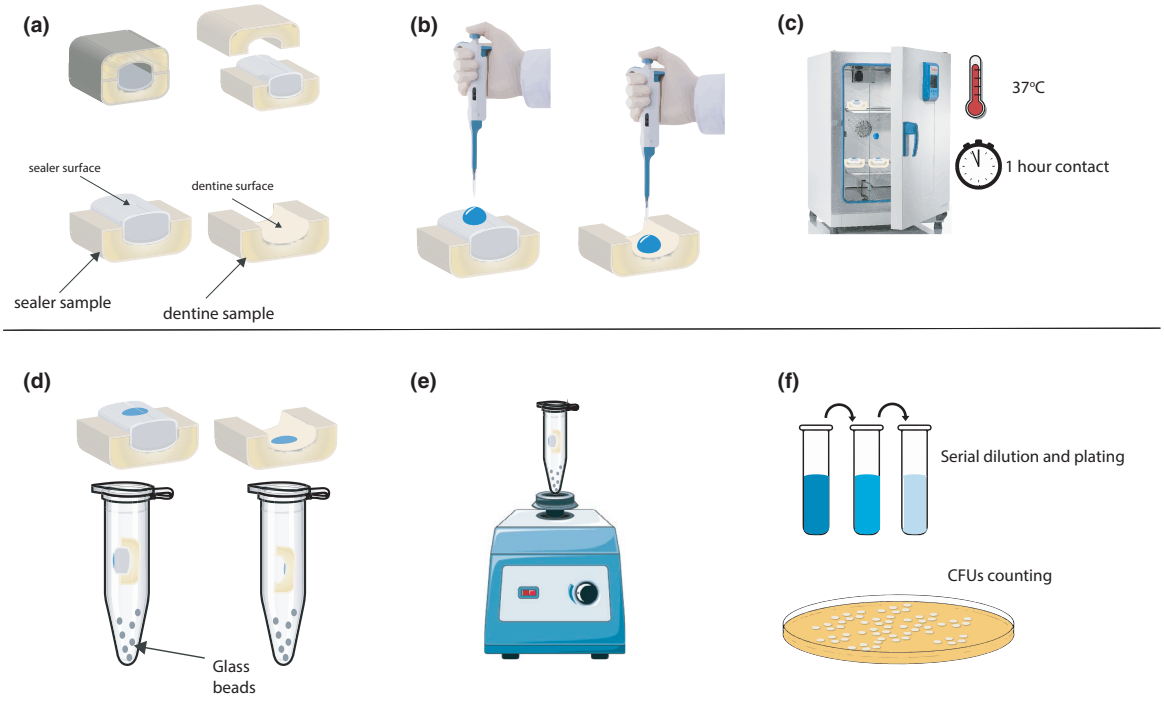
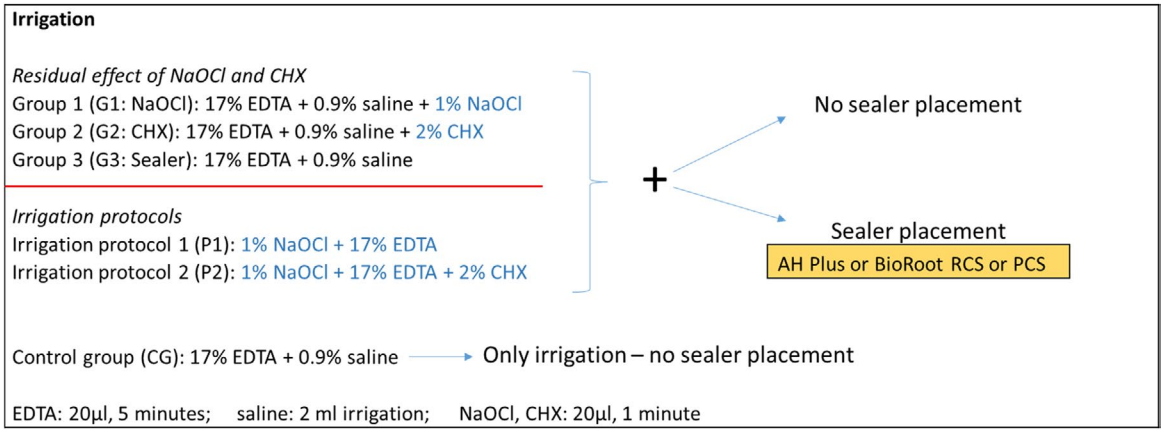


FIGURE 2 Schematic representation of planktonic assay. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer was adhered to one segment whilst the adjacent segment was macroscopically free of sealer remnants (a). An amount of 5 μl *E. faecalis* bacterial suspension was carefully placed upon the dentine (dentine samples) and the sealer surface (dentine-sealer sample), or only upon the dentine surface in irrigation groups without sealer and control group (b). The specimens were incubated at 37°C for 1 h, whilst complete evaporation of the suspension's liquid was inspected (c). The sealer samples and their adjacent dentine samples were separately transferred in vials containing 500 μl PBS and were vigorously vibrated with glass beads (d and e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO_2 supplemented atmosphere (f)

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



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

Internal validity of split tooth model—evaluation of dentine surfaces

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

To assess the type of failure on the sealer-dentine interface (adhesive: complete separation of sealer from dentine, cohesive: rupture of material bulk within the sealer, or a mix) and identify any sealer remnants on dentinal walls, scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) were performed. Briefly, two root blocks for each sealer were mounted on aluminium stubs, carbon coated (Agar Scientific), and viewed with the scanning electron microscope (TM4000Plus, Hitachi). Accelerating voltage ranged between 5 and 15 kV and the probe current between 125 and 300 pA. High magnification EDS chemical analysis was carried out at 15 kV and a working distance of 8.5 mm. Scanning electron micrographs at high magnification in the backscatter electron mode were captured, and EDS was performed in selected spots and rectangular areas of the samples. Furthermore, elemental maps at the same levels were performed and each element was marked out/designated in a different colour. EDS was also performed over sealers prepared in circular samples to define their elemental profile. At this point, it is emphasized that the analysis regards root blocks that were incubated for 24 h, when the sealers were at the most premature stage of setting compared to 7 and 28 days set materials and therefore were more prone to deform during separation of tooth segments leading to possible cohesive type of failure. Moreover, all root blocks were pre-treated with 17% EDTA for 5 min aiming for constant background of dentinal tubules, as smear layer did not allow to distinguish between tooth structure and sealer remnants.

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

Antibacterial assays

Bacteria and media

The antibacterial properties of both dentine and dentine-sealer surfaces were assessed on the previously described *ex vivo* tooth model. *Enterococcus faecalis* American Type Cell Culture Collection (ATCC) 19434 was grown overnight for 18 h in Tryptone Soya Broth (TSB) at 37°C, 5% CO_2 supplemented atmosphere. The bacteria were suspended in PBS to an optical density at 600 nanometres (OD_{600}) of 1.0, corresponding to approximately 2×10^8 Colony Forming Units (CFU)/ml (Figure 3a). The

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

Planktonic bacteria—Direct Contact Test (DCT)

An amount of 5 μl from *E. faecalis* suspension was carefully placed upon the dentine (dentine sample) and the dentine-sealer surface (dentine-sealer sample), and only upon the dentine surface in irrigation groups without sealer placement and control group (Figure 2b). The specimens were incubated at 37°C for 1 h, whilst complete evaporation of the suspension's liquid was inspected (Figure 2c). The dentine-sealer samples and their adjacent dentine samples were separately transferred into vials containing 500 μl PBS and were vigorously vibrated with glass beads (Figure 2d,e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO_2 supplemented atmosphere (Figure 2f). Carryover effect of the method was also assessed. Briefly, an amount of 5 μl from the bacterial suspension was placed into vials containing 500 μl PBS together with dentine and dentine-sealer samples derived from all groups. These samples were vigorously vibrated with glass beads. Possible carryover effect was measured after serial dilutions and CFUs were calculated as described previously. Experiments for potential carryover effect were performed in triplicate.

Bacteria in biofilms—Direct Contact Test (DCT)

Membrane filters (MF-Millipore™ Membrane Filter, 0.45-μm pore, Merck) were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 μl of each bacterial inoculum OD_{600} 1.0 was applied upon the outer surface of membranes (Figure 3a,b). The agar plates were incubated at 37°C in a 5% CO_2 supplemented atmosphere for 48 h and mono-species biofilms were established (Figure 3c). The biofilm formation was verified with the use of confocal laser scanning microscopy (Figure 3d). The filter membranes were positioned upon the dentine and sealers with the established biofilms facing their surfaces (Figure 4b). The specimens were wrapped with Parafilm M to secure the membrane filters upon the surfaces and placed at 37°C in a 5% CO_2 supplemented atmosphere for 24 h (Figure 4c). After incubation time, the Parafilm M was removed, and a droplet of 10 μl sterile distilled water was transferred upon the membranes to enable a gentle detachment from the sealer and dentine. Each membrane with its corresponding dentine or dentine-sealer sample was

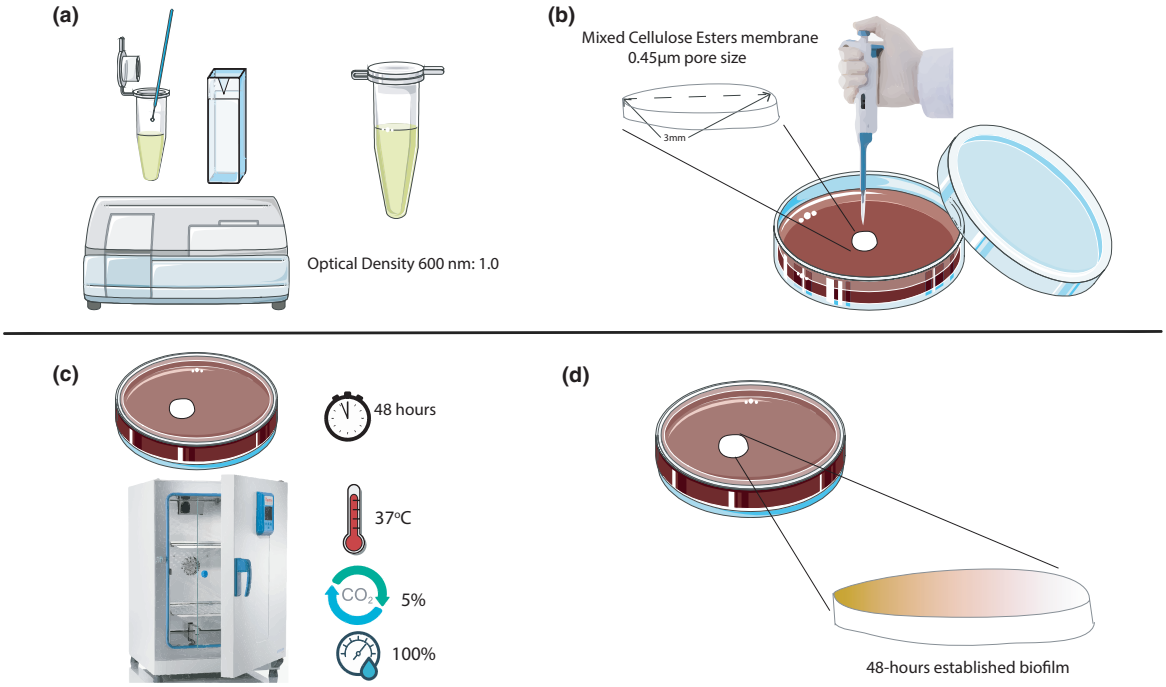


FIGURE 3 Schematic representation of biofilm formation. The bacteria were suspended in PBS to an optical density at 600 nanometres (O600) of 1.0 (a). Membrane filters were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 μl of each bacterial inoculum OD600 1.0 was applied upon the outer surface of membranes (b). The agar plates were incubated at 37°C in a 5% CO_2 supplemented atmosphere for 48 h (c). The monospecies biofilms were established and verified with the use of confocal laser scanning microscopy (d)

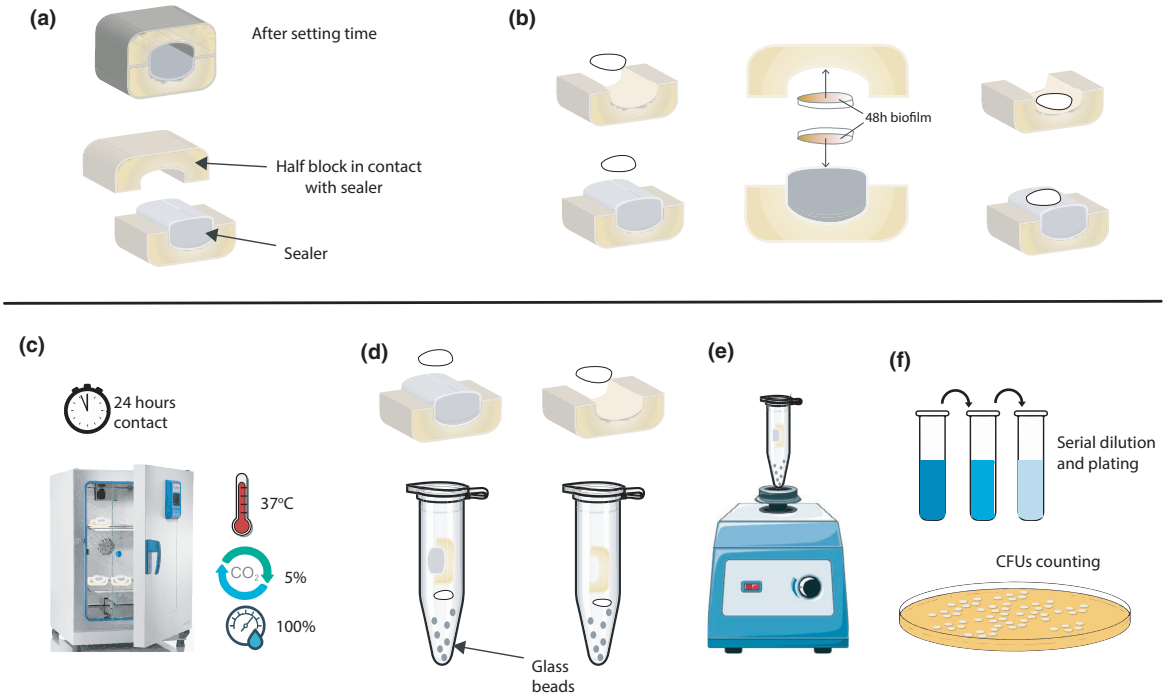


FIGURE 4 Schematic representation of biofilm assay. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer was adhered to one segment whilst the adjacent segment was macroscopically free of sealer remnants (a). The filter membranes were positioned upon the dentine and sealers with the established biofilms facing their surfaces (b). The specimens were wrapped with Parafilm M to secure the membrane filters upon the surfaces and placed at 37°C in a 5% CO_2 supplemented atmosphere for 24 h (c). After 24 h, each membrane with its corresponding sealer-dentine or sealer block was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads (c and d). After serial dilutions in PBS, colony forming units (CFU) were counted after incubation at 37°C in a 5% CO_2 supplemented atmosphere (f)

transferred to vials containing 5 ml PBS (Figure 4d) and vigorously vortexed with glass beads (Figure 4e). After serial dilutions in PBS, CFUs were counted after incubation at 37°C in a 5% CO_2 supplemented atmosphere (Figure 4f). Carryover effect of the method was also assessed. Filter membranes with established biofilms served as positive controls and were placed in vials containing 5 ml PBS. Dentine and dentine-sealer samples derived from all groups were put in the same vial. These samples were vigorously vibrated with glass beads. Possible carryover effect was measured after serial dilutions and CFUs were calculated as described previously. Experiments for potential carryover effect were performed in triplicate.

Statistical analysis

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

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

7 and 28 days).

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

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

RESULTS

Internal validity of tooth model

SEM examination showed adhesive mode of failure at the sealer-dentine interface. Sealer residues could be sporadically identified, but no full dentine coverage was evident in any of the surfaces investigated. A full series of SEM micrographs with elemental analysis is presented in Figure S1. AH Plus bonded to dentine with sealer tags and after separation process the whole bulk of the material was debonded. Only few sealer tags rich in Zr could be identified in dentinal tubules (Figure 5a). BioRoot RCS demonstrated trace elements on dentine without full coverage (Figure 5b). As for PCS, elemental analysis showed few sealer tags rich in Zn (Figure 5c). Thus, the model was considered reproducible as the SEM examination of dentine samples indicated consistent separation between dentine and dentine-sealer surfaces. This finding enabled to proceed further with antibacterial assays, testing both the dentine and dentine-sealer surfaces.

Antibacterial properties of dentine and dentine-sealer surfaces

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

Planktonic E. faecalis*, dentine surfaces*

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

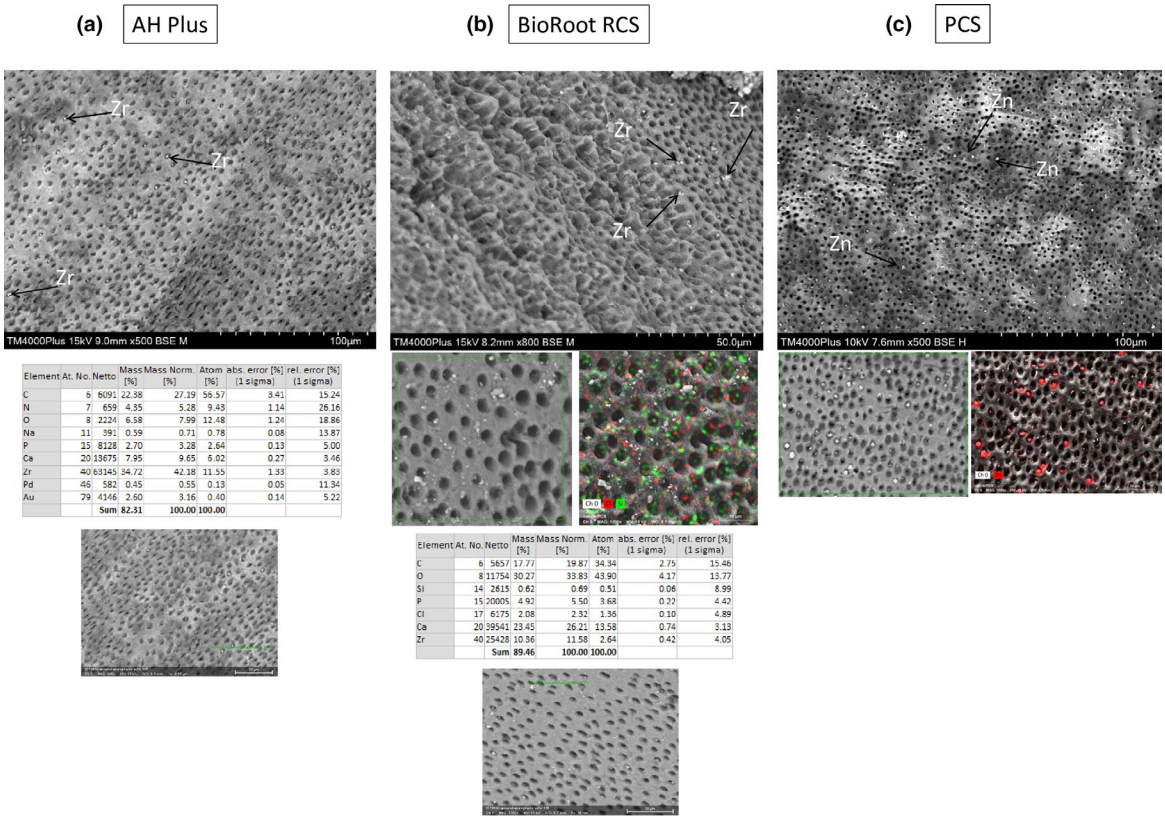


FIGURE 5 Representative scanning electron micrographs of dentine having been in contact with the tested sealers retrieved from the split tooth model: AH Plus (a), BioRoot RCS (b) and PCS (c). The black arrows indicate sealer residues (white circular spots) rich in Zr for AH Plus/BioRoot RCS and Zn for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

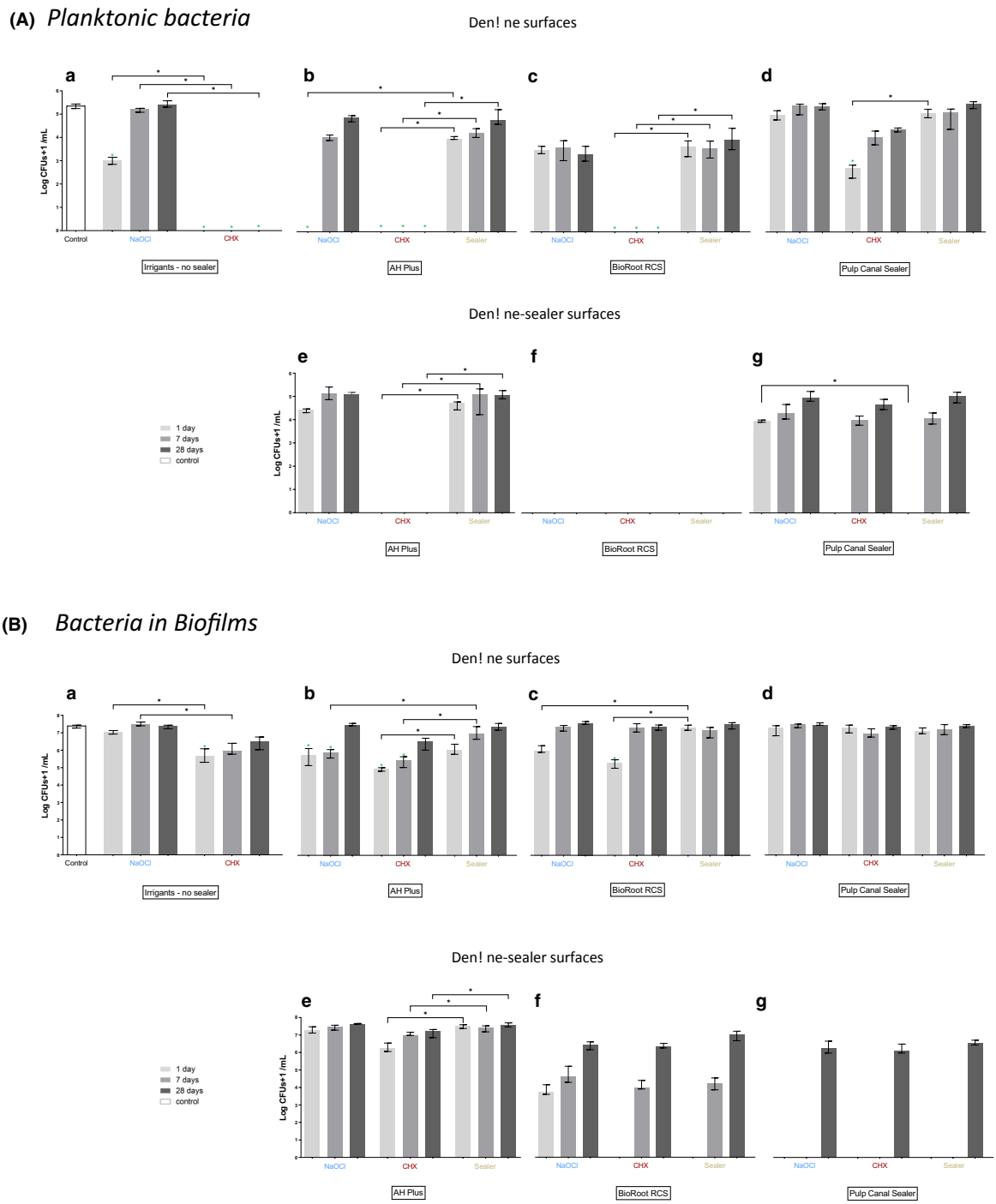


FIGURE 6 Residual effect of NaOCl and CHX on dentine and dentine-sealer surfaces. Median Log (CFU + 1)/ml and 25–75 interpercentile range (error bars) of *E. faecalis* in planktonic forms (A) and in biofilms (B) after direct contact with each dentine and dentine-sealer surface. For dentine surfaces,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

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

Planktonic E. faecalis*, dentine-sealer surfaces*

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

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

E. faecalis *in biofilms, dentine surfaces*

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

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

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

Effect of irrigation protocols on dentine and dentine-sealer surfaces

Planktonic E. faecalis*, dentine surfaces*

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

Planktonic E. faecalis*, dentine-sealer surfaces*

No surviving *E. faecalis* bacteria were retrieved from BioRoot RCS surfaces which have been in contact with dentine treated both with P1 and P2 (Figure 7Af). When CHX was used as the last irrigant (P2), AH Plus surfaces which have been in contact with dentine eliminated *E. faecalis* and significantly reduced its numbers compared to AH Plus in contact with dentine treated with P1 for all three irrigation times ($p < .05$; Figure 7Ae). PCS surfaces in contact with dentine treated with P1 eliminated *E. faecalis* after 1 day incubation, whilst treatment with P2 did after both 1 and 7 days incubation.

E. faecalis *in biofilms, dentine surfaces*

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

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

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

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

Multiple regression analyses

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

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

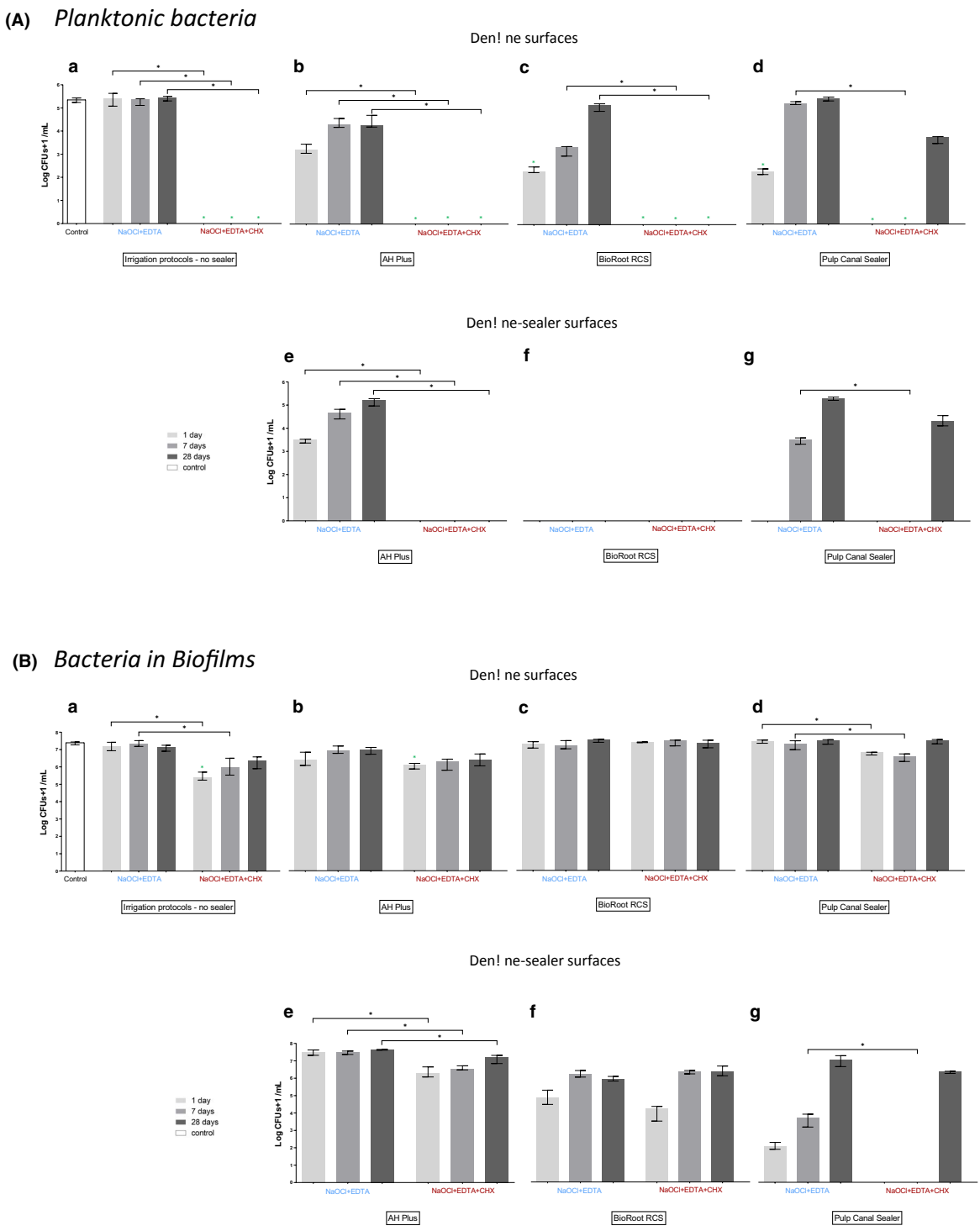


FIGURE 7 Antibacterial effect of two irrigation protocols on dentine and dentine-sealer surfaces. Median Log (CFU + 1)/ml and 25–75 interpercentile range (error bars) of *E. faecalis* in planktonic forms (A) and in biofilms (B) after direct contact with each dentine and dentine-sealer surface. For dentine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

The multiple regression model (2) statistically significantly predicted bacterial survival (CFUs), $F(5, 642) = 110.426$, $p < .001$, adj. $R^2 = .46$. All five variables added statistically significantly to the prediction, $p < .001$. Models' fit, regression coefficients and standard errors for both models (1) and (2) can be found in Table S4. Models' fit, regression

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

To assess the isolated effect of 1% NaOCl and 2% CHX on antibacterial properties, the smear layer was removed with the use of 17% EDTA and the root blocks were rinsed in between with saline solution to avoid any additional interactions between EDTA and NaOCl-CHX (Rossi-Fedele et al., 2012). As clinical procedures most often entail the use of several irrigation liquids, two relevant irrigation protocols were also tested: 1% NaOCl + 17% EDTA and 1% NaOCl + 17% EDTA + 2% CHX. Only treatment with CHX, both in group 2 and in irrigation protocol 2, eliminated the planktonic bacteria on dentine surfaces in all incubation times up to 28 days. This result corroborates earlier literature on CHX's ability to possess long-lasting antibacterial properties due to substantivity (Carrilho et al., 2010; Rosenthal et al., 2004; Souza et al., 2018). In this study, 1% NaOCl had inferior antibacterial properties to 2% CHX that can be potentially attributed to its low concentration; *in vitro* studies indicate that higher percentage of NaOCl could result in increased antibacterial properties (Gomes et al., 2001; Tirali et al., 2009). However, clinical findings suggest no significant differences in antimicrobial properties of NaOCl in different concentrations (0.5%–5.25%; Byström & Sundqvist, 1985; Soares & Pires Júnior, 2006). Moreover, a recent randomized clinical study reported similar clinical outcomes for high (5%) and low (1%) NaOCl concentrations (Verma et al., 2019). Toxicity of NaOCl to periapical tissues as well as its deleterious effect on the integrity of dentine structure and on the collagen matrix is concentration dependent, with higher concentrations being more irritating (Farook et al., 2014; Marending et al., 2007; Pashley et al., 1985; Zancan et al., 2021). Thus, in our study, 1% NaOCl was preferred to higher percentages as low NaOCl concentrations have been shown to combine both antimicrobial properties and low cytotoxicity. Application of CHX (G2 and P2) managed to reduce significantly the numbers of *E. faecalis* in biofilms only after 1-day incubation period, confirming that biofilms are more resistant than their planktonic counterparts (Bjarnsholt, 2013).

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

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

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

In this study, PCS exhibited antibacterial properties mainly on sealer surfaces, which had been in contact with dentine and high efficacy against *E. faecalis* biofilms. This indicates that PCS may exhibit moderate constant antibacterial properties, related to the gradual release of eugenol (Hauman & Love, 2003; Marchese et al., 2017), given that in biofilm assays the contact time of dentine or dentine-sealer surfaces with bacteria was 24 h. In addition, a new study demonstrated a decrease in *E. faecalis* live bacteria upon PCS surfaces after an initial biofilm formation, which may be correlated to the release of zinc (Long et al., 2020). On the contrary, the antibacterial effect of the PCS upon dentine was weak especially against biofilms. This could be attributed to the pronounced shrinkage that PCS displays stored at 100% humidity (Camilleri & Mallia, 2011), which might lead to loose (non-tight) contact with the dentinal walls and thus compromised antibacterial properties. Moreover, a zinc-oxide eugenol impression material exhibited reduction in dimensions after disinfection with aqueous CHX and NaOCl solutions (Amin et al., 2009). Previous studies on zinc oxide eugenol cements as PCS have demonstrated improved antibacterial activity after mixing with CHX (Nambu, 1984; Tchaou et al., 1996). In our study, treatment with CHX (G2 and P2) conferred antibacterial properties on dentine walls against planktonic *E. faecalis*.

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

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

CONCLUSIONS

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

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CONFLICT OF INTEREST

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

ETHICAL APPROVAL

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

AUTHOR CONTRIBUTIONS

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

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III

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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

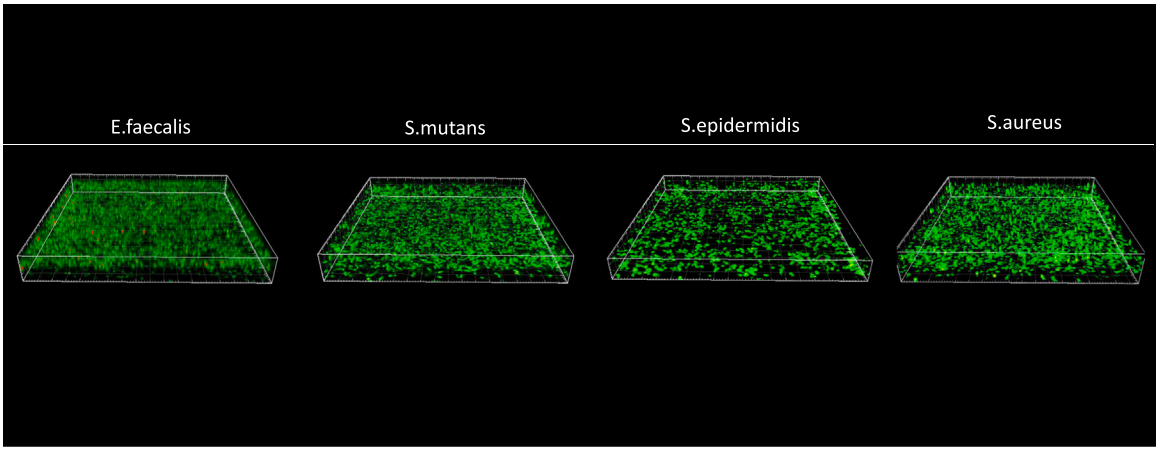

Fig. 1 – Representative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-h monospecies biofilms grown on polyester coverslips. The scanning was performed from the top of the biofilm to the membrane surface using a 60 × water lens, 0.5 µm step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

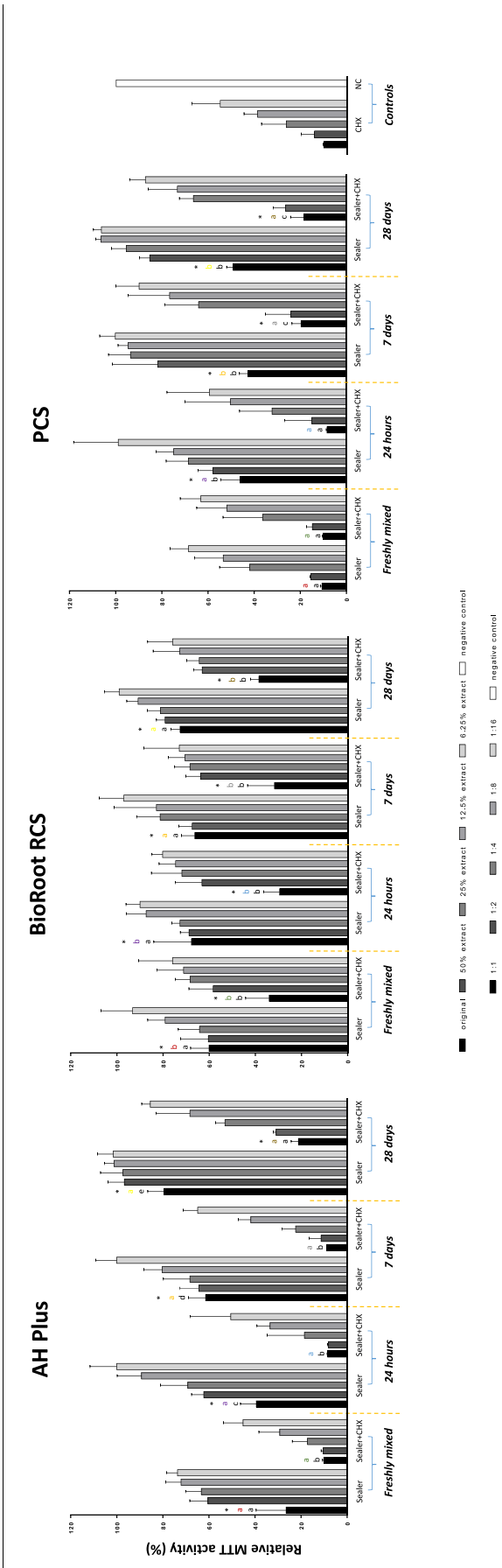

Fig. 2 – Mean relative MTT activity and standard deviation of L929 murine fibroblasts after 24-h exposure to undiluted sealer leachates and their dilutions. Statistical analysis was performed in undiluted leachates. The statistical differences between the leachates of each group and CHX control are denoted 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.

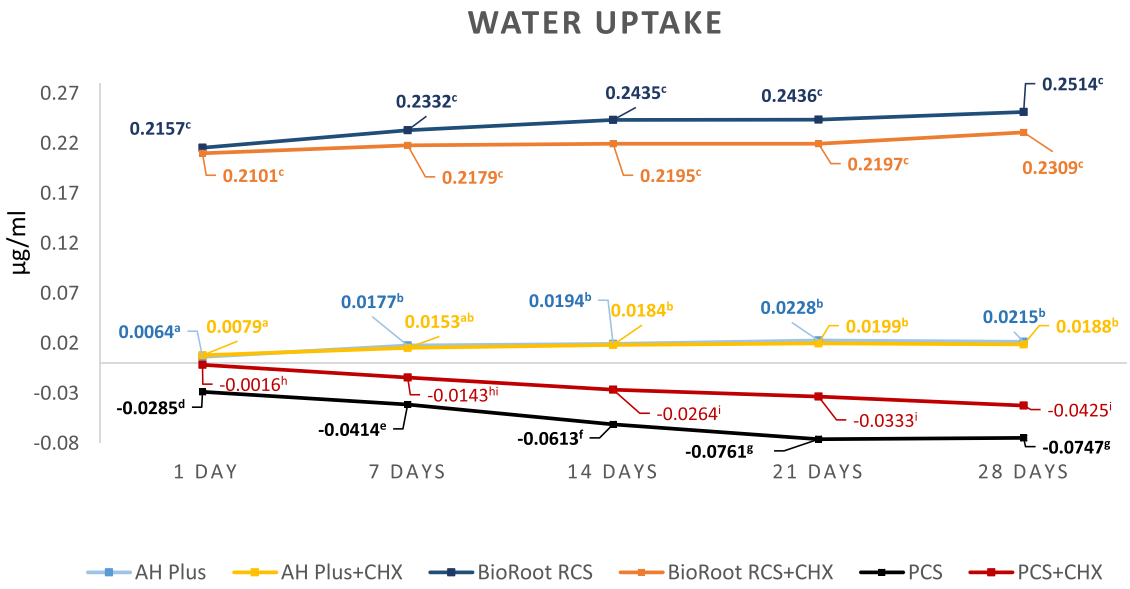

Fig. 3 – Mean water uptake values for test sealers with and without CHX contact. Read horizontally (within the same sealer and experimental condition, between different immersion periods, Tukey's multiple comparison test) and vertically (within the same immersion period, between different sealers and 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}

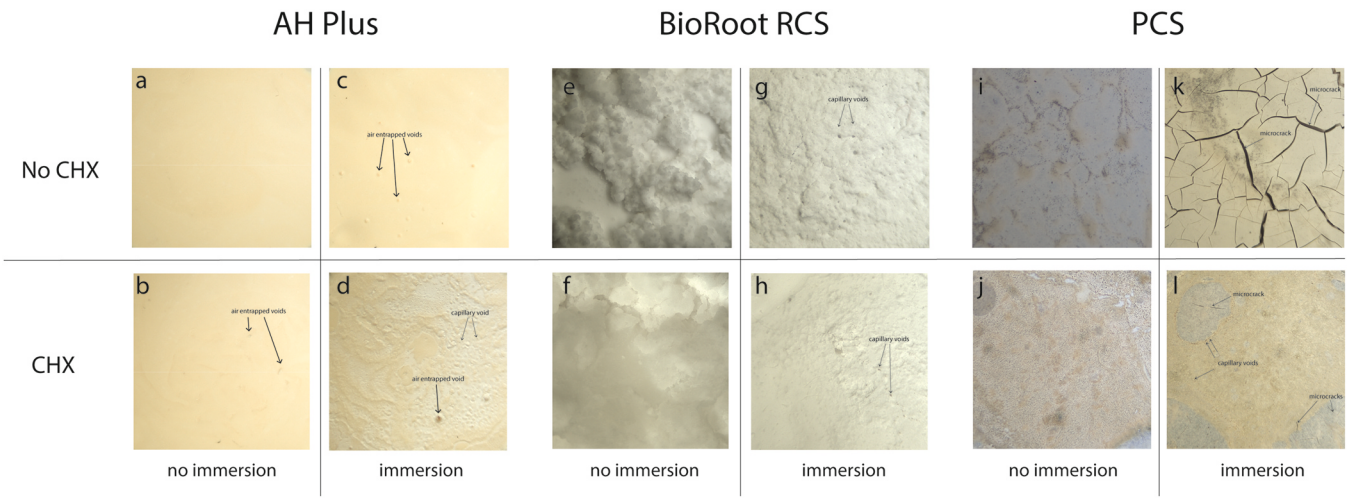

Fig. 4 – Representative microscopical images of the immersed or non-immersed sealer surfaces showing different features including air entrapped voids, capillary voids, crystal-like formations: (a) AH Plus, no immersion, no CHX; (b) AH Plus, no immersion, CHX; (c) AH Plus, immersion, no CHX; (d) AH Plus, 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

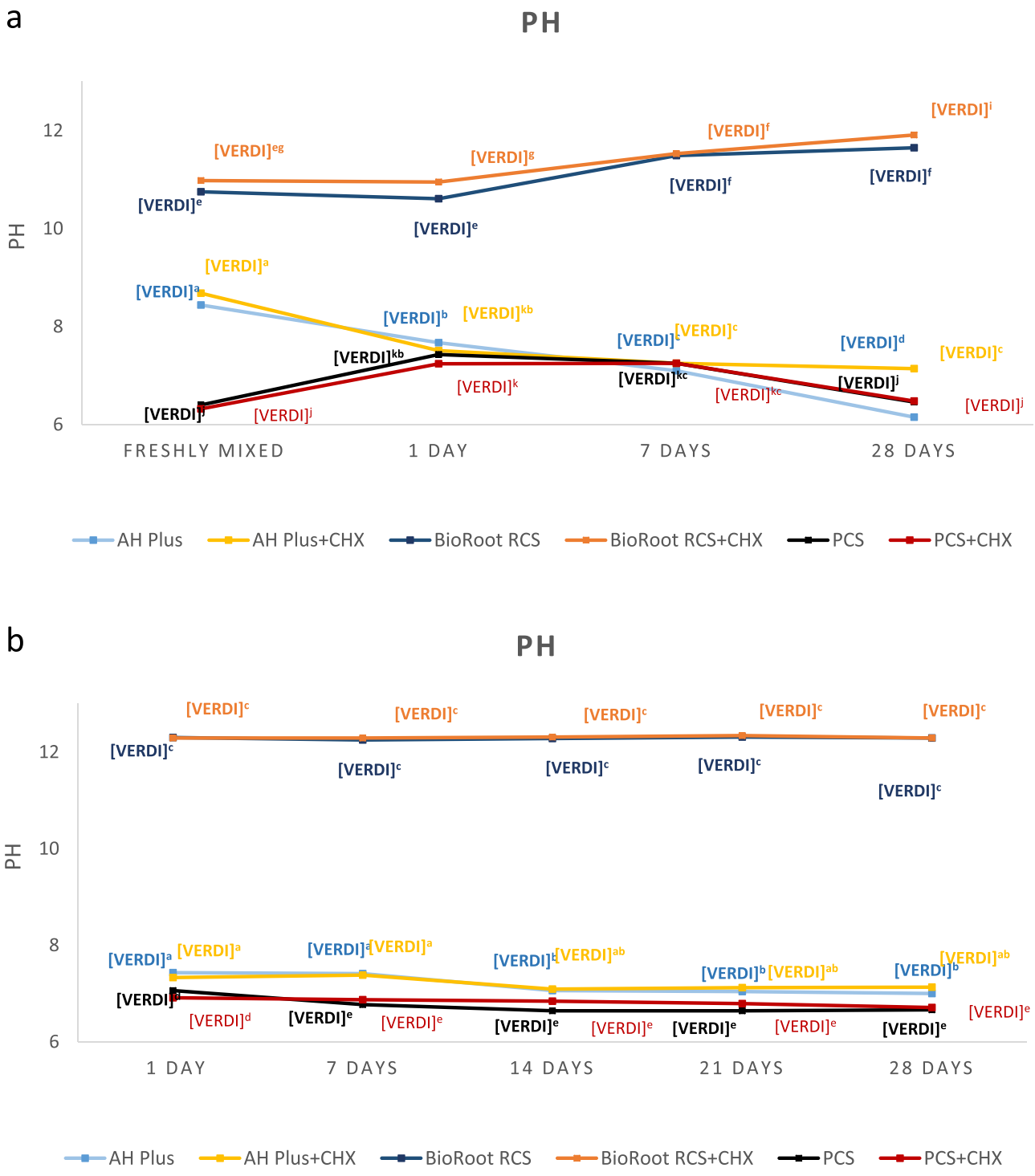

Fig. 5 – Mean pH values of freshly mixed, 24 h, 7 days and 28 days set sealers' leachates (for biological properties) in contact or not with CHX (pH = 5.98 ± 0.11). Saline 0.9% (pH = 5.6 ± 0.09) used as the extraction vehicle (a). Mean pH values of sealers' leachates in contact or not with CHX (pH = 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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Erratum

'Erratum to "Effect of chlorhexidine digluconate on antimicrobial activity, cell viability and physicochemical properties of three endodontic sealers" [Dent Mater 38 (2022) 1044–1059]'

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The publisher regrets that in the above referenced article a late correction to replace Fig. 5 was not implemented. The correct Fig. 5 is now represented accurately below.

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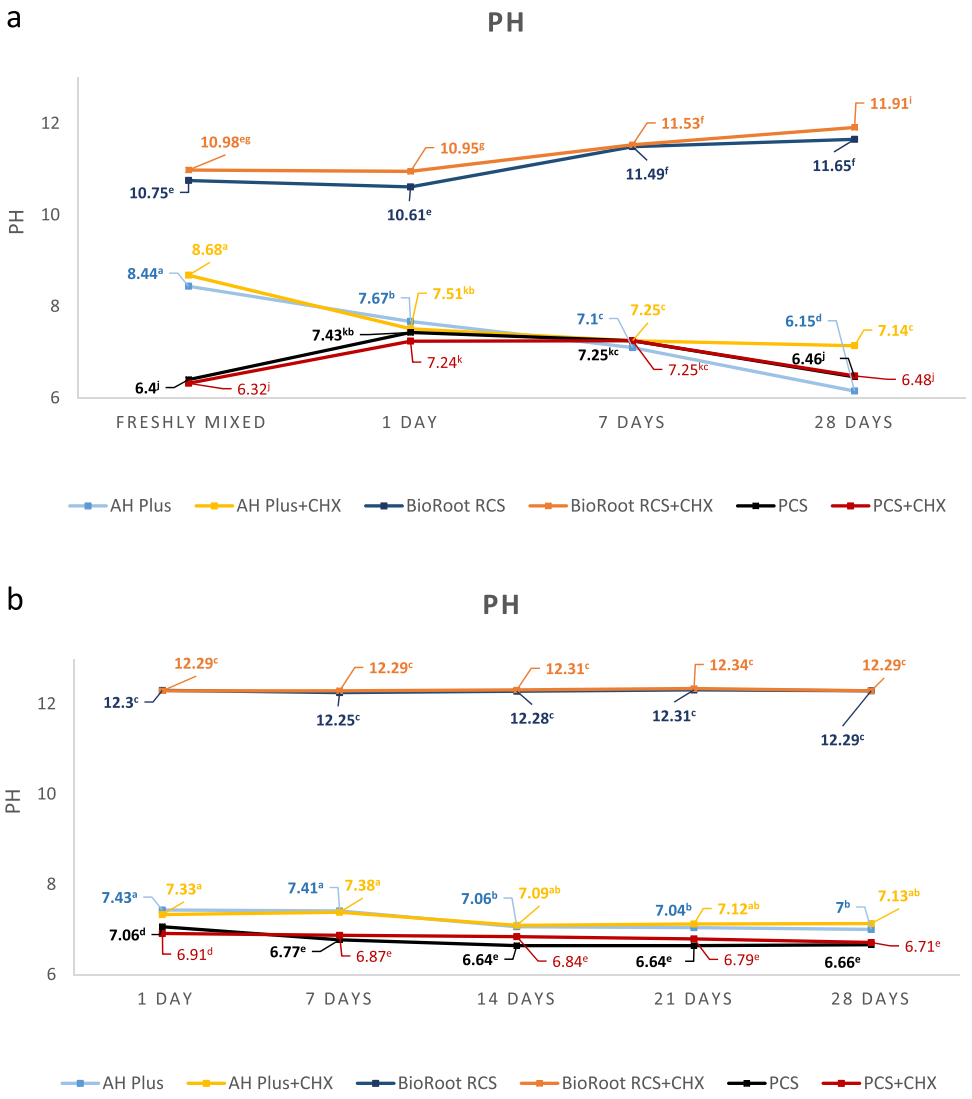

Fig. 5 – Mean pH values of freshly mixed, 24 h, 7 days and 28 days set sealers' leachates (for biological properties) in contact or not with CHX (pH = 5.98 ± 0.11). Saline 0.9% (pH = 5.6 ± 0.09) used as the extraction vehicle (a). Mean pH values of sealers' leachates in contact or not with CHX (pH = 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, $p > 0.05$.

Errata list

Doctoral candidate: Vasileios Kapralos
Titel of thesis: Interactions Between Irrigation Fluids and Endodontic Sealers: Assessing Biological, Physicomechanical and Chemical aspects

Abbreviations for different types of corrections:
Cor – correction of language
Cpltf – change of page layout or text format

Page/Line/Footnote	**Original text**	**(type of correction) Corrected text**
i/11/	"…a 6-months…"	(Cor): "… a 6-month …"
i/20/	"…Pia, you made me to stay…"	(Cor): "… Pia, you made me stay …"
i/29/	"…would be..."	(Cor): "…would have been…"
i/32/	"…for every single person…"	(Cpltf/Cor): "…to each and every person …"
ii/9-10/	"…the School of Dentistry in Birmingham staff and the students and PhD candidates…"	(Cpltf/Cor): "… the staff of the School of Dentistry in Birmingham as well as the students and PhD candidates …"
ii/27/	"…the most fascinating scientific milestones we haven't yet achieved, they are the ones to come…"	(Cpltf/Cor): "…the most fascinating scientific milestones are those we haven't yet reached, they are the ones yet to come..."
ii/28/	"…I would like to dedicate the original poem to all	(Cpltf/Cor): "…I would like to dedicate the original poem to

	the contributors to science...”	all those who contribute to science...”
ii/30-33/	“…The most beautiful sea hasn’t been crossed yet. The most beautiful child hasn’t grown up yet. Our most beautiful days we haven’t seen yet. And the most beautiful words I wanted to tell you I haven’t said yet….”	(Cpltf/Cor): "…The most beautiful sea has not yet been crossed. The most beautiful child hasn't grown up yet. We haven't seen our best days yet. And the most beautiful words I wanted to say to you, I haven't said yet…”
ix/header/	“…summary of papers…”	(Cpltf): The header was removed
ix/page number/	right positioning	(Cpltf): left positioning
ix/1/	“…physicochemical characterization…”	(Cor): “… physicochemical characterisation…”
ix/14/	“…Surface characterization …”	(Cor): “… Surface characterisation …”
ix/19/	“…The individualization …”	(Cor): “… The individualisation …”
x/26/	“…water sorption, solubility and…”	(Cpltf/Cor): “…water sorption, solubility, and…”
xiii/contents/		(Cpltf): New blank page (page 50) was added which altered the paging in the rest of the manuscript. Page numbers in the contents have been now adjusted accordingly.
2/16/	“…utilizing…”	(Cor): “…utilising…”

4/24-26/	"…But these species derived from uncommon phyla are not high-abundance members of the endodontic community …"	(Cpltf/Cor): "…Nevertheless, species derived from uncommon phyla are not high-abundance members of the endodontic community …"
6/33/	"…chronic infections, controlled inflammation and limited…"	(Cpltf/Cor): "…chronic infections, controlled inflammation, and limited…"
7/7/	"…organization…"	(Cor): "…organisation…"
8/27/	"…colonize…"	(Cor): "…colonise…"
12/6/	"…gram-negative bacteria and…"	(Cpltf/Cor): "…gram-negative bacteria, and…"
13/3/	"…gram-negative bacteria and…"	(Cpltf/Cor): "…gram-negative bacteria, and…"
14/header/	"…Endodontic Sealers…"	(Cpltf): "…1. Introduction…"
14/2-3/	"…demineriliz**i**ng…"	(Cor): "…demineril**i**sing."
14/3-4/	"…demineriliz**i**ng…"	(Cor): "…demineril**i**sing."
14/page number/	right positioning	(Cpltf): left positioning
15/1/	"…resin based sealers…"	(Cor): "…resin-based sealers…"
15/31/	"…no long term antimicrobial…	(Cor): "…no long-term antimicrobial…
16/header/	"…Irrigation in Endododntics…"	(Cpltf): "…1. Introduction…"

16/page number/	right positioning	(Cpltf): left positioning
16/22/	"…sterilized…"	(Cor): "…sterilised…"
17/header/	"…Irrigation in Endododntics…"	(Cpltf): "…Endodontic sealers…"
17/12/	"…polymerization…"	(Cor): "…polymerisation…"
17/15/	"…polymerization…"	(Cor): "…polymerisation…"
17/23-24/	"…zirconium oxide, silica and iron oxide pigments. AH Plus paste B contains amines, silica and silicone oil…"	(Cpltf/Cor): "…zirconium oxide, silica, and iron oxide pigments. AH Plus paste B contains amines, silica, and silicone oil…"
20/30/	"…by the swiss dentist…"	(Cor): "…by the Swiss dentist…"
22/header/	"…Interactions between chlorhexidine and endodontic sealers…"	(Cpltf): The header was removed
22/page number/	right positioning	(Cpltf): remove paging
24/2/	"…characterization…"	(Cor): "… characterisation…"
26/3/	"… standardized…"	(Cor): "… standardised…"
27/13/	"… jeopardize…"	(Cor): "… jeopardise…"
27/16/	"… standardized…"	(Cor): "… standardised…"
33/3/	"…such as cell viability, proliferation, apoptosis,	(Cpltf/Cor): "…such as cell viability, proliferation, apoptosis, adhesion,

	adhesion, morphology and gene expression…"	morphology, and gene expression…"
34/header/	"Sealer surfaces and leachates"	(Cpltf): "3. Methodological aspects"
34/page number/	right positioning	(Cpltf): left positioning
35/7/	"… jeopardize…"	(Cor): "… jeopardise…"
35/10/	"… analyzed…"	(Cor): "… analysed…"
38/36/	"…specimens and also tested directly…"	(Cpltf/Cor): "…specimens and is also tested directly…"
39/header/	"Tooth model"	(Cor): "Tooth models"
39/20/	"…characterization…"	(Cor): "… characterisation…"
40/header/	"Tooth model"	(Cpltf): "3. Methodological aspects"
40/3/	"…characterized…"	(Cor): "… characterised …"
40/Figure legend, Fig 3.9/	"…characterized…"	(Cor): "… characterised …"
40/ page number/	right positioning	(Cpltf): left positioning
41/header/	"Tooth model"	(Cor): "Tooth models"
41/5/	"…and analyzed as…"	(Cor): "…and analysed as…"
41/7/	"…utilizing…"	(Cor): "…utilising…"
41/24/	"… standardized…"	(Cor): "… standardised…"

41/32/	"… dichotomized…"	(Cor): "… dichotomised…"
42/Figure legend, Fig 3.10/	"… standardized…"	(Cor): "… standardised…"
42/Figure legend, Fig 3.10/	"… dichotomized…"	(Cor): "… dichotomised…"
43/header/	"Tooth model"	(Cor): "Tooth models"
43/18/	"… emphasized…"	(Cor): "… emphasised…"
45/header/	"3. Methodological aspects"	(Cpltf): "Tooth models"
45/page number/	left positioning	(Cpltf): right positioning
46/header/	"Statistical methods"	(Cpltf): "3. Methodological aspects"
46/page number/	right positioning	(Cpltf): left positioning
47/3/	"...minimizing…"	(Cor): "…minimising…"
47/12/	"… standardized…"	(Cor): "… standardised…"
47/23/	"...analyzed…"	(Cor): "…analysed…"
50/new page/		(Cpltf/Cor): New blank page and change of paging (numbering) in the following pages by one number (applies to all remaining pages of the thesis). For the rest of this Errata list the page numbers will be referring to the new pages. For example, page 51 is

		the old page 50. It will be now designated as 51 (50old) etc…
51(50old)/header/	“4. Discussion”	(Cpltf): header erased
51(50old)/page number/	left positioning	(Cpltf): right positioning
52(51old)/4/	“…These results points…”	(Cor): “…These results point…”
53(52old)/header/	“4. Discussion”	(Cpltf): “Sealer surfaces and leachates”
53(52old)/page number/	left positioning	(Cpltf): right positioning
54(53old)/5/	“…were setting time-dependent…”	(Cor): “…were setting time dependent…”
55(54old)/header/	“4. Discussion”	(Cpltf): “Sealer surfaces and leachates”
55(54old)/page number/	left positioning	(Cpltf): right positioning
56(55old)/18/	“…showing favorable results …”	(Cor): “…showing favourable results …”
56(55old)/19/	“…Moreover the low…”	(Cor): “…Moreover, the low…”
57(56old)/header/	“4. Discussion”	(Cpltf): “Sealer surfaces and leachates”
57(56old)/page number/	left positioning	(Cpltf): right positioning
57(56old)/4/	“…more hydrophillic after…”	(Cor): “…more hydrophilic after…”

59(58old)/header/	“4. Discussion”	(Cpltf): “Findings particular to the split tooth model”
61(60old)/header/	“5. Concluding remarks”	(Cpltf): header erased
61(60old)/page number/	left positioning	(Cpltf): right positioning
61(60old)/20/	“…characterization…”	(Cor): “… characterisation…”
63(62old)/header/	“6. Future perspectives”	(Cpltf): header erased
63(62old)/page number/	left positioning	(Cpltf): right positioning
65(64old)/23/	“… jeopardize…”	(Cor): “… jeopardise…”
67(66old)/page number/	left positioning	(Cpltf): right positioning
67(66old)/header/	“Bibliography”	(Cpltf): header erased
68(67old)/ref 26/	“Journal of Dental Research”	(Cpltf/Cor): “J Dent Res”
69(68old)/ref 41/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
74(73old)/ref 126/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
74(73old)/ref 127/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
75(74old)/ref 153/	“ØRSTAVIK D”	(Cpltf/Cor): “Ørstavik D”
75(74old)/ref 153/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
77(76old)/ref 187/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
77(76old)/ref 188/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”

77(76old)/ref 189/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
77(76old)/ref 190/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
77(76old)/ref 191/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
77(76old)/ref 192/	“Journal of the American Dental Association”	(Cpltf/Cor): “J Am Dent Assoc”
77(76old)/ref 193/	“Australian Dental Journal”	(Cpltf/Cor): “Aust Dent J”
77(76old)/ref 194/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 195/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 196/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 197/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
78(77old)/ref 200/	“Journal of Dentistry”	(Cpltf/Cor): “J Dent ”
78(77old)/ref 201/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
78(77old)/ref 202/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 205/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 206/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”

78(77old)/ref 207/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
78(77old)/ref 208/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
78(77old)/ref 209/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 210/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
78(77old)/ref 211/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
79(78old)/ref 212/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
79(78old)/ref 213/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
79(78old)/ref 215/	“The Saudi dental journal”	(Cpltf/Cor): “Saudi Dent J”
79(78old)/ref 216/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
79(78old)/ref 223/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
79(78old)/ref 224/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
79(78old)/ref 225/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
79(78old)/ref 226/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
79(78old)/ref 227/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
79(78old)/ref 228/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”

80(79old)/ref 229/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
80(79old)/ref 230/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
80(79old)/ref 231/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
80(79old)/ref 232/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
80(79old)/ref 233/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
80(79old)/ref 234/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
80(79old)/ref 235/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
80(79old)/ref 237/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
80(79old)/ref 238/	“Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology”	(Cpltf/Cor): “Oral Surg Oral Med Oral Pathol Oral Radiol Endod”
80(79old)/ref 244/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
81(80old)/ref 256/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
81(80old)/ref 257/	“Dental Traumatology”	(Cpltf/Cor): “Dent Traumatol”
81(80old)/ref 258/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
81(80old)/ref 260/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
81(80old)/ref 264/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”

82(81old)/ref 270/	“Dental Materials”	(Cpltf/Cor): “Dent Mater”
82(81old)/ref 274/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
82(81old)/ref 280/	“Brazilian Oral Research”	(Cpltf/Cor): “Braz Oral Res”
83(82old)/ref 289/	“International Journal of Biomaterials”	(Cpltf/Cor): “Int J Biomater”
83(82old)/ref 293/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
83(82old)/ref 294/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
83(82old)/ref 297/	“Journal of Applied Biomaterials and Functional Materials”	(Cpltf/Cor): “J Appl Biomater Funct Mater”
83(82old)/ref 298/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
84(83old)/ref 300/	“Dentistry Today”	(Cpltf/Cor): “Dent Today”
84(83old)/ref 305/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”
85(84old)/ref 320/	“Journal of Dental Research”	(Cpltf/Cor): “J Dent Res”
85(84old)/ref 321/	“Journal of Dental Research”	(Cpltf/Cor): “J Dent Res”
85(84old)/ref 328/	“Dental Materials Journal”	(Cpltf/Cor): “Dent Mater J”
85(84old)/ref 330/	“Oral Surgery, Oral Medicine, Oral Pathology”	(Cpltf/Cor): “Oral Surg Oral Med Oral Pathol Oral Radiol Endod”

85(84old)/ref 332/	“Journal of Endodontics”	(Cpltf/Cor): “J Endod”
85(84old)/ref 333/	“The Journal of the American Dental Association”	(Cpltf/Cor): “J Am Dent Assoc”
87(86old)/ref 355/	“Oral Surg Oral Med Oral Pathol”	(Cpltf/Cor): “Oral Surg Oral Med Oral Pathol Oral Radiol Endod”
87(86old)/ref 357/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
87(86old)/ref 363/	“Oral Surg Oral Med Oral Pathol”	(Cpltf/Cor): “Oral Surg Oral Med Oral Pathol Oral Radiol Endod”
88(87old)/ref 374/	“Scientific Reports”	(Cpltf/Cor): “Sci Rep”
89(88old)/ref 393/	“Endodontic Topics”	(Cpltf/Cor): “Endod Topics”
93(92old)/ref 462/	“International Endodontic Journal”	(Cpltf/Cor): “Int Endod J”