Exploration of Degradative Activities of 
*Enterococcus faecalis* and Determinants of Bacterial 
Biofilm Proliferation within the Sealer-Dentin 
Interfacial Margins

By

Muna Qasim Mustafa Marashdeh

A thesis submitted in conformity with the requirements 
for the degree of Doctor of Philosophy
Graduate Department of Dentistry 
University of Toronto

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Exploitation of Degradative Activities of *Enterococcus faecalis* and Determinants of Bacterial Biofilm Proliferation within the Sealer-Dentin Interfacial Margins

Muna Qasim Marashdeh

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Abstract

**Background:** Root canal treatment involves disinfecting and sealing the root canal system. The interface between the root canal sealer and dentin might degrade over time, allowing for interfacial microbial biofilm proliferation and passage to the periradicular tissues and treatment failure. Salivary and bacterial enzymes may accelerate this degradation process. One of the commonly detected bacteria in the failed root canal treated teeth is *Enterococcus faecalis*. **Objectives:** 1) To investigate the effect of simulated human salivary esterases (SHSE) on the sealer-dentin interfacial integrity, by measuring the depth of interfacial bacterial biofilm proliferation, 2) To investigate the effect of SHSE on the physical properties of root canal sealers, 3) To assess the esterase-like degradative enzymatic activities of *E. faecalis* and their effect on methacrylate resins, and 4) To assess the collagenase-like enzymatic activities of *E. faecalis* and their degradative effect on dentin. **Methods:** Root canal treated specimens were incubated in SHSE for up to 360 days followed by cultivation of *E. faecalis* biofilm in constant
medium fermenter, mimicking pathogenic intraoral growth conditions. Confocal laser scanning microscopy was used to assess the depth of interfacial bacterial biofilm proliferation. *E. faecalis* esterase and collagenolytic-like activities were measured and their effect on the methacrylate resins and dentinal collagen matrix was evaluated.

**Results:** SHSE increased the sealer-dentin interfacial degradation as measured by the increased bacterial biofilm proliferation, and had an adverse effect on some of the physical properties of the root canal sealers. *E. faecalis* showed hydrolase, esterase-like and collagenase-like activities in levels that degraded methacrylate resins and dentinal collagen, respectively. **Conclusions:** This study demonstrated the potential effect of the salivary and bacterial enzymes on the outcome of root canal treatment; hydrolase activities could increase the sealer-dentin interface degradation, and may facilitate bacterial migration from the root canal into the periapical area, compromising the root canal treatment. Development of esterase resistant, and anti-collagenolytic sealers and/or treatment could be used to mitigate the effect of the salivary and bacterial enzymes.
Acknowledgements

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<tr>
<td>AS</td>
<td>Aggregation substance</td>
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<tr>
<td>BC</td>
<td>Bioceramic sealer</td>
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<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>BisEMA</td>
<td>Ethoxylated Bisphenol A Glycol Dimethacrylate</td>
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<td>BisGMA</td>
<td>Bisphenol-Glycidyl-Dimethacrylate</td>
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<tr>
<td>BisHP PPP</td>
<td>Bis-Hydroxy-Propoxy-Phenyl-Propane</td>
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<tr>
<td>BTC</td>
<td>Butyrylthiocholine</td>
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<tr>
<td>CBBF</td>
<td>Chemostat Based Biofilm Fermenter</td>
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<td>CE</td>
<td>Cholesterol Esterase</td>
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<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EfaA</td>
<td>Enterococcus faecalis antigen A</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>ER</td>
<td>Epoxy Resin Sealer</td>
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<td>Esp</td>
<td>Enterococcal surface protein</td>
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<td>GP</td>
<td>Gutta-percha</td>
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<tr>
<td>HEMA</td>
<td>2-Hydroxyethyl Methacrylate</td>
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<td>HIN</td>
<td>Heat Inactivated Bacteria</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<td>MSCRAMM</td>
<td>Microbial Surface Component Recognizing Adhesive Matrix Molecules</td>
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<tr>
<td>MTA</td>
<td>Mineral Trioxide Aggregate</td>
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<tr>
<td>o/n</td>
<td>Overnight</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCE</td>
<td>Pseudocholine Esterase</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>pNPA</td>
<td>p-nitrophenyl Acetate</td>
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<tr>
<td>pNPB</td>
<td>p-nitrophenyl Butyrate</td>
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<tr>
<td>RC</td>
<td>Resin Composite</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>SE</td>
<td>Self-Etch Adhesive</td>
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<td>SHSE</td>
<td>Simulated Human Salivary Esterases</td>
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<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<td>TE</td>
<td>Total-Etch Adhesive</td>
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<tr>
<td>TGDMA</td>
<td>Triethylene Glycol Dimethacrylate</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>UDMA</td>
<td>Urethane Dimethacrylate</td>
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<tr>
<td>ZOE</td>
<td>Zinc Oxide-Eugenol</td>
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Preface

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Toronto. The research described herein was conducted in the Department of Biomaterials between September 2013 and April 2017. This work is to the best of my knowledge original, except where acknowledgments and references to previous work are made.

- Dissertation format
  Chapter 1: A general introduction, hypotheses, and objectives of the current project.
  Chapter 2: Detailed literature review of the topics pertaining to the research problem.
  Chapters 3-6: Compilations of the experimental data that are submitted for publication. They are presented in their submitted form with minor changes to include more details. All papers planned to be submitted to the Journal of Endodontics.
  Chapter 7: A general discussion of all the experimental data obtained in the study.
  Chapter 8: Conclusions, recommendations, and future directions
  Chapter 9: Supplementary data in the study that was not included in the publications

- Scholarships/ Awards/ conferences reproduced from the current project:
  2017 IBBME (Institute of Biomaterials and Biomedical Engineering) Scientific day (poster presentation)
  2017 IADR (International Association for Dental Research General Meeting 2017), San Francisco; poster presentation
  2017 Dentistry Research Day; poster presentation (2nd place Award)
  2016 IBBME Scientific day (poster presentation)
  2016 SGS (School of Graduate Studies/ University of Toronto) Conference Travel Award
  2016 AAE-16 (American Association of Endodontics Annual Meeting, April, 2016), San Francisco; Oral presentation
  2016 Dentistry Research Day; Oral presentation (Finalist three oral presentations)
  2013-2017 Graduate Studies Scholarship, Faculty of Dentistry, Jordan University of Science and Technology (JUST).
Chapter 1

Introduction

1.1 Background

Endodontic infections are caused by microbial ingress into the pulp and periradicular tissues [1, 2]. The rationale for endodontic treatment is to eradicate the infection, prevent microorganisms from infecting, or re-infecting the root, and/or periradicular tissues, and to preserve the structural integrity of the root canal [2-4]. Because of the invasion of bacteria into the dentinal tubules and formation of biofilms, and because of anatomic irregularities of root canal systems, the conventional disinfection regimens used clinically are only partially effective, resulting in residual bacteria and positive-growth cultures in 10% to 70% of canals [5-8].

Of the teeth receiving endodontic treatment, a reported 11% do not heal [9] and require further treatment, preferably root canal retreatment. The most recent information available on the frequency of endodontic procedures in USA was obtained from the American Dental Association Survey of Dental Services Rendered, conducted in late 2005-early 2006 and published in August of 2007. It is estimated that 22.3 million endodontic procedures are performed annually; the estimated total of all endodontic procedures increased about 10% from 1999 to 2006 (from 20.3 million to 22.3 million), while no matching data is available from Canada. Considering that the US population is estimated at 324,752,779 by the end of March 2017 (US Census Bureau, www.census.gov/main/www/popclock.html) and the Canadian population at 36,535,118 (Statistics Canada, www.statcan.gc.ca), we can infer that the number of endodontic treatments performed in Canada is in the range of 2.48 million/year.

According to the Ontario Dental Association’s fee guide for Endodontists, the cost of endodontic (root canal) treatment is $649.65 and $1221.72 for single- and multi-rooted teeth, respectively. Averaging the cost of single- and multi-rooted treatment, the financial burden for endodontic treatment in Canada is estimated approximately $2.3 billion/year. The Ontario Dental Association fees for retreatment are $843.59 and $1674.56 for single-
and multi-rooted teeth, respectively. Considering that 11% of 2.48 million endodontically treated teeth require retreatment, and averaging the cost of single- and multi-rooted retreatment, suggests that the financial burden for endodontic retreatment in Canada is approximately $341 million/year. This considerable burden highlights the importance of minimizing the proportion of teeth that require retreatment.

Endodontic infections, both primary and secondary are biofilm-mediated infections [10-12], therefore it is important to understand the pathogenic potential of the root canal microbiota to form the basis for proper disinfection [13]. Primary endodontic infections are caused by microorganisms that initially invade the pulp tissue and are associated with large variety of bacterial species [14], in contrast to secondary or persistent infections that are caused by microorganisms entering or persisting in the root canal after the initial treatment (failed endodontic treatment) and have less variable microbiota. Enterococcus faecalis is a microorganism commonly detected in asymptomatic, persistent endodontic infections. Its prevalence in such infections ranges from 24% to 77% [15]. It is able to survive in the root canal as a single organism or as a major component of the flora [16].

Infected root canals are conventionally filled after chemo-mechanical preparation [17]. The core material Gutta-percha (GP) in association with different nonbonding (do not bond to root canal dentin and root canal core) endodontic sealers has been used for long time as a traditional root canal filling system [18]. GP’s primary function is to occupy space, while the sealer’s properties are more important to provide a tight seal [19]. The surrounding oral environment affects the physiochemical properties of different root canal sealers, and hence their clinical performance might vary accordingly [20-22].

Several endodontic sealers are available commercially; AH Plus is an epoxy-based resin sealer that is widely used among the endodontic community due to its favorable physical and biological properties [23-25]. However it lacks the ability to bond to the core material and the tooth structure. New sealers are constantly being developed in attempts to provide better properties by improving the bonding to the tooth, and/or to improve biocompatibility and/or improve antibacterial properties. Methacrylate resin-based root
canal sealers were suggested with the intent to overcome the drawbacks of the traditional sealers by bonding to both root canal dentin as well as the core material, forming a sealer-filler monoblock that has been suggested to reduce bacterial ingress pathways and strengthen the root to some extent [26-28]; however, their apparent improvement did not result with anticipated better outcome [25]. Bonding methacrylate resins to root dentin is unpredictable [29]. Polymers of resin materials are bound by unprotected ester linkages that are susceptible to hydrolysis [30, 31], consequently resin-dentin interface undergoes degradation over time [32-34]. Bioceramic-based sealers (BC) are newly introduced sealers to the endodontic field in line with the increased use and the positive outcome of bioceramic technology in medicine and dentistry [23, 35]; the material is biocompatible [36], contains calcium phosphate, which improves the interaction of these materials with dentin (the mineral infiltration zone) [37, 38], requires moisture to set [39], while contamination with blood and saliva might adversely affect its performance [40, 41].

Degradation at sealer-dentin interface leads to salivary and microbial proliferation over time [42]. Salivary and bacterial esterases can catalyze this process and accelerate the degradation of the resin-dentin interface [33, 43, 44]. A host-derived family of proteolytic enzymes known as matrix metalloproteinases (MMPs), found both in saliva and dentin [45-47] has been shown to be involved in the degradation of the unprotected collagen fibrils within the hybrid layer [34, 48]. Once activated, these peptidases are responsible for the intrinsic auto-degenerative process of dentinal degradation [34, 49]. Some bacterial collagenases demonstrated the ability to degrade collagen in a manner similar to human MMPs [50], and therefore they could potentially accelerate the dentinal collagen degradation, affecting the bond to tooth dentin, and increasing the possibility of root facture [32, 51].

The relationship between simulated salivary and bacterial enzymes and the dentin-sealer interface degradation will be the main focus in this study.
1.2 Purposes of the study

“Research questions: Will simulated human salivary esterases (SHSE) affect the marginal break down at the sealer-root dentin interface, and the physical properties of root canal sealers? Will this effect be dependent on the period of SHSE exposure? Can esterase-like activities from E. faecalis degrade dental methacrylate resins? And can collagenolytic activities from E. faecalis degrade dentinal collagen, and contribute to the marginal degradation of the sealer-dentin interface?”

Root canal fillings are intended to prevent microbial proliferation in the canal after treatment. The dentin-sealer interface degrades over time, allowing for bacterial biofilm colonization of root filled teeth and treatment failure. The surrounding environment could adversely affect the physicochemical properties of root canal sealers and dentin; therefore the outcome of the root canal treatment could be affected. One aim of this study is to assess the effect of SHSE on the biostability of the sealer-root dentin interface, and the physical properties of root canal sealers. A second aim is to explore the effect of bacterial esterases and collagenolytic activities on the biostability of methacrylate resins and root dentin, respectively. E. faecalis is used as a biological measure for the quality of the restoration’s interfacial margins by measuring the degree of the bacterial proliferation into the compromised interfaces, formation of biofilms within the interface, and the live/dead bacterial ratio outcome. In addition, the effect of SHSE on the physical properties of root canal sealers, and E. faecalis ability to degrade (hydrolyze) resin composite and dentinal collagen is assessed. The study provided valuable information for the development of endodontic sealer systems and their clinical applications.
1.3 Hypotheses and Objectives

1.3.1 Hypotheses

1. *E. faecalis* biofilm proliferation depth and viability along the sealer-dentin interfacial margins are modulated by sealer type, and SHSE exposure.

2. SHSE exposure of root canal sealers will adversely affect the physical properties of the methacrylate resin based materials but not the epoxy resin and bioceramic based sealers.

3. *E. faecalis* possesses esterase-like activities that degrade dental methacrylate resin composites, self-etch adhesive, and total etch adhesive.


1.3.2 Objectives

1. To assess the effect of sealer type, and SHSE exposure on *E. faecalis* biofilm proliferation depth and viability using chemostat-based biofilm fermenter (CBBF) and confocal laser scanning microscopy (CLSM).

**Rationale:** Saliva contains cholesterol esterase (CE) and pseudocholine esterase-like (PCE) hydrolase activities [52], both activities showed the ability to degrade methacrylate-based resin dental composites and adhesives [52], decrease their bond to dentin [43, 53], and increase bacterial biofilm proliferation between the restoration-dentin interfaces [33]. The CBBF allows for continuous controlled flow of media therapy (Brain heart infusion (BHI)) simulating in vivo pathogenic oral conditions to cultivate *E. faecalis* biofilm [33, 42]. Using CLSM to characterize the salivary enzyme catalyzed degradation of resin-dentin interface enables the view of fully intact specimens, with little to no sample disruption when compared to other microscopy techniques.
2. To assess the effect of SHSE exposure for up to 4 weeks on the surface microhardness, compressive strength, dimensional change, and solubility of bioceramic sealer (BC), epoxy resin sealer (ER), and resin composite (RC).

Rationale: The chemical and physical properties of root canal sealers have an effect on the quality of root canal filling and consequently on the outcome of the endodontic treatment [21, 22]. Sealers come into contact with the oral physiological solutions such as blood and/or saliva [54-57]. Moisture can have a crucial effect on the setting process of sealers [41]. SHSE adversely affected the depth of interfacial *E. faecalis* biofilm proliferation in the first objective in some material groups; therefore it was critical to assess its effect on the physical properties of the root canal sealers used in the first objective.

3. To measure SHSE-like activities from *E. faecalis* and assess the effect of these activities on the degradation of methacrylate-based resin composite, total-etch, and self-etch adhesives

Rationale: Methacrylate resins have the potential to be used as a root canal sealers, but their use nowadays in root canal is mainly limited to post cementation [58, 59], and coronal restorations of endodontically treated teeth [60]. Methacrylate resin polymers are prone to hydrolysis due to the presence of unprotected ester linkages; salivary and bacterial esterases can catalyze this process and increase the degradation of the resin-dentin interface at a greater rate [33, 43, 44]. *E. faecalis* enters the root-filled canal via coronal leakage during or after root-canal treatment as secondary invaders [61]. Confirming *E. faecalis* esterases activities and their ability to degrade dental methacrylate resins will help to explain its ability as a coronal invader to penetrate through the methacrylate-tooth interface.

4. To measure the protease, collagenolytic-like activities of *E. faecalis*, and its ability to degrade human dentinal collagen matrix.

Rationale: Collagen is the major protein found in dentin, it constitutes ~90% of the organic matrix, most of which is type I collagen [62]. Collagen preserves the
structural, mechanical, and functional integrity of the dentin [63]. Bacterial infection caused by coronal leakage is a significant contributor to endodontic failure [64]. Bacterial enzymes may contribute to the degradation of collagen fibrils and deterioration of structural integrity in root dentin with time [63]. *E. faecalis* has been frequently detected in root canal samples from patients with persistent endodontic infections [65-67]. It enters the root-filled canal via coronal leakage during or after root-canal treatment as secondary invaders [61]. The ability of *E. faecalis* to cause infections has been linked to numerous virulence factors [68]. Measuring *E. faecalis* collagenolytic activity could explain its ability to degrade the dentinal collagen matrix, and potentially compromise the interface and tooth structure. The collagenolytic activity may facilitate the migration of *E. faecalis* and other species from the root canal into the periapical lesion and may explain its role in apical periodontitis.
Chapter 2

Literature review

2.1 Endodontic infection

In 1965, Kakehashi et al. [1], showed conclusively that pulpal and endodontic problems are primarily related to microbial contamination of the root canal system, thus periapical endodontic disease can only occur in the presence of microorganisms [69]. Since that time endodontology has increasingly focused on the ways of eliminating microorganisms from the entire root canal system. Therefore a thorough understanding of the endodontic microbiota associated with different forms of disease is the basis for the success of endodontic treatment [4].

Chemomechanical preparation of the root canal is performed in order to reduce bacterial concentrations. It has been found that microbes still remain even after thorough chemomechanical preparation of the root canal system [7, 70]. Follow-up studies of endodontic treatment have shown a higher rate of healing when bacterial culture obtained prior to root filling yielded no visible growth [71]; when teeth had positive cultures at the time of filling only 68% healed, while 94% of the teeth healed when the culture was negative [72].

2.2 Endodontic microbiology

The use of molecular approaches to analyze the endodontic microbiota improved the sensitivity (the ability to detect low levels of bacteria) [73], and understanding of the etiology of the endodontic disease compared to the classic culture-based microbiological methods [74, 75]. Secondary endodontic infections had been known to have less diverse and complex microbiota when compared to primary infections [76], while recent research using advanced molecular techniques showed no significant difference in the microbial diversity between primary and secondary infections [77, 78]. Endodontic infections contain many microorganisms that are not cultivable but may contribute to a significant degree in the pathogenesis of disease and resistance to treatment [79].
Morphological studies of the endodontic microbiota in untreated (primary infection) and treated (persistent/secondary infection) teeth have demonstrated that the dominant pattern of bacterial colonization of the root canal system resembles that of biofilm [10-13].

Biofilm can be defined as a sessile multicellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substance (EPS). Typically, bacteria occupy 10-20% of the total volume of the biofilm [13, 80]. The extracellular matrix mainly consists of polyanionic exopolysaccharides secreted by bacteria itself [17], proteins, nucleic acids (extracellular DNA) and salts making up to 80-90% of the biofilm volume [80]. The EPS can act as a diffusion barrier, preventing toxic substances such as antibiotics and disinfectants from reaching their target [80-82].

Biofilm infections are responsible for 65-80% of the human infections in the developed world [82]. Phenotypic characteristics of biofilm cells are markedly different from their planktonic counterparts [83, 84]. In comparison to planktonic growth, biofilms demonstrate greatly enhanced surface adherence properties [85], lowered susceptibility to anti-microbial agents [86], and host-immune defense mechanisms [87].

The first stage of biofilm formation (Fig. 2.1) includes adhering of free floating cells (planktonic) to a surface, then the cells aggregate to form micro-colonies and excrete extracellular polymeric substances (EPS). The micro-colonies progressively enlarge and coalesce to form multiple layers of cells pile up on the surface forming a mature biofilm characterized by the presence of macro-colonies surrounded by water channels that help distribute nutrients and signaling molecules. Finally, to survive when nutrients become limited, or simply to spread and colonize to other niches, some biofilm cells can detach individually or in clumps. The molecular mechanisms that regulate the distinct developmental steps in biofilm formation vary greatly between different bacterial species, and even depend on the environmental conditions for the same given species [80, 83, 88, 89].
In dentistry dental plaque is the most known type of biofilm [90], it has been defined as “a specific but highly variable structural entity consisting of microorganisms and their products embedded in a highly organized intercellular matrix”. Biofilms have been implicated also in the development of dental caries and periodontal disease [91]. In endodontics, the identification of biofilm was earlier reported by Nair 1987 [92], and can be divided into intracanal, external root (cementum), periapical biofilms, and biomaterial centered biofilms (Fig. 2.2) [93].

**Intracanal biofilms**: These biofilms are formed by multiple species of microorganisms in the root canal dentin of the infected tooth. They degrade the dentin substrate as a consequence of the interaction of bacteria and their metabolic products on dentin. Most of the organisms existed as loose collections of filaments, spirochetes, coccis, and rods [13].

**External root (cementum) biofilms**: Biofilms that are formed on the root (cementum) surface adjacent to the apex of endodontically infected teeth. *Fusobacterium nucleatum, Porphyromonas gingivalis*, and *Tannerella forsythensis* were found to be associated with extraradicular biofilm [95].

**Periapical biofilms**: Isolated biofilms located in the periapical region of endodontically infected teeth. A limited diversity of species has been reported in periapical biofilms. These include members of *Actinomyces* and *Propionibacterium propionicum* that produce “sulphur granules” [96].

**Biomaterial-centered biofilms**: Arise from adherent bacteria on contaminated artificial biomaterials, such as filling materials; GP may become contaminated with bacteria that lead to adherent biofilms [97, 98]. Dental clinicians encounter a great challenge trying to control and eradicate the biofilm-associated diseases. Introduction of the biofilm concept to endodontic microbiology was the major step forward to the understanding of root canal infections, especially those of the persistent kind.
Figure 2.1: Schematic representation of the microbial biofilm development stages. The first stage in biofilm formation includes initial attachment of the cells to a surface, followed by cells aggregation and formation of a monolayer along the surface and microcolonies in the second stage. The third step includes the formation of a mature community with mushroom-shaped macro-colonies. During the third stage, the biofilm structure can be disrupted, and microbial cells can be liberated and transferred into another location/surface, causing expansion of the infection.

Figure 2.2: Schematic diagram of the Endodontic bacterial biofilm classification.
2.2.1 Microbial community in primary endodontic cases

Primary endodontic infections are associated with a large variety of bacterial species [14] dominantly anaerobic bacteria, along with some facultative anaerobic bacteria [74, 99]. Overall, the bacterial density per canal varies from $10^3$ to $10^8$ [100, 101]. In primary endodontic cases, root canal environment provides better nutritional supply rich with peptides and amino acids for bacterial inhabitants of root canal system. This environment favors the growth of anaerobic proteolytic species [102]. Black-pigmented bacteria are the species frequently isolated from primary endodontic cases and apical abscesses, due to their proteolytic activity [103]; the most prevalent species isolated from primary endodontic infections are *Prevotella intermedia*, *Prevotella nigrescens*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* [104]. *Fusobacterium nucleatum*, *Veillonella parvula*, and *Eubacterium* are also strongly related with primary endodontic cases [105].

2.2.2 Microbial community in secondary and persistent endodontic cases

Secondary infections are caused by microorganisms that enter the root canal at some time after professional intervention, while persistent infections are caused by microorganisms that persisted in the canal after the primary or secondary infections [76]. Unlike primary infections, the microbial flora of secondary or persistent periapical periodontitis is less variable mainly composed of Gram-positive facultative anaerobic bacteria [106-108]. This selection process is due to the harsh environmental conditions in the instrumented and medicated canals [109]. Recent molecular techniques demonstrated that microbiota of persistent infections is more complex than previously anticipated by culture studies [77], and the bacterial community profiles in treated cases vary from individual to individual [110].

Microbiological findings from filled root canals with persistent periapical disease have shown a high proportion of Enterococci and *Candida albicans* [111, 112]. Some characteristics of yeasts are common with Enterococci; both can survive as a monoinfection and invade dentinal tubules [113]. Beside Enterococci a high prevalence
of Streptococci followed by Lactobacilli, *Actinomyces* sp., Peptostreptococci, *Eubacterium alactolyticus*, *Propionibacterium propionicum*, *Dialister pneumosintes* and *Filifactor alocis* was reported [114].

*Actinomyces* is a well-known pathogen found in therapy-resistant retreatment cases. *A. israelii* and *A. meyerii* are more frequently found and involved in periapical actinomycosis. *Propionibacterium propionicum* is a facultative anaerobic organism; it is a normal resident of the oral cavity and has also been found in persisting intraradicular and extra-radicular endodontic infections that do not respond to conventional endodontic treatment [115].

2.2.2.1 *Enterococcus faecalis*

Up to 90% of Enterococcal infections in humans are caused by *E. faecalis*. *E. faecalis* is a Gram-positive bacterium that can occur singly, in pairs, or as short chains. It is facultative anaerobe that possesses the ability to grow in the presence or absence of oxygen [116]. It has gained attention by its ability to persist after root canal treatment and has been isolated from both primary and secondary infections, although secondary cases are about nine times more likely to harbor *E. faecalis* than primary infections [116-118]. *E. faecalis* is highly detected in root canal infections treated in multiple visits and/or in teeth left open for drainage [61, 119]. The prevalence of *E. faecalis* in root canals was associated with its presence in saliva [67]. The genotype similarity between *E. faecalis* isolates from saliva, the pulp chamber, and the root canal [120] suggest that it is a secondary coronal invader that succeeds in colonizing the canal and resists treatment [76]. *E. faecalis* is not part of the commensal oral flora, its origin remains unclear [61], and the isolates from the root canals were not related to those from the normal gastrointestinal microflora [121].

The survival of *E. faecalis* after endodontic therapy has been attributed to many virulence factors [15, 68, 117, 122], such as aggregation substance, gelatinase, cytolysin toxin, extracellular superoxide production, capsular polysaccharides [123-125], and the ability to penetrate into dentinal tubules in as little as 48 h of inoculation [126] (Fig 2.3). *E. faecalis* is highly resistant to intracanal dressings [127]; one of the most important is
calcium hydroxide. The antimicrobial effect of calcium hydroxide is related to the high pH that it produces, and *E. faecalis* can withstand this because of a proton pump in its cytoplasmic membrane [123]. Moreover, it is reported to be capable of forming biofilms in root canals, which can greatly enhance its tolerance to antimicrobial regimens [128]. When *E. faecalis* was grown on abiotic surfaces such as microtiter plates, it was found that its ability to develop into biofilms was dependent on the surface attributes of the substratum and that this could vary according to the environmental and nutritional conditions present [129].

Recently the status of *E. faecalis* as the main causative agent of endodontic treatment failures has been questioned; *E. faecalis* has not been detected in all secondary infections with apical periodontitis [116, 130], it is rarely one of the most dominant species in root canal–treated teeth, other microorganisms might be more dominant like *Fusobacterium nucleatum* [131], *Porphyromonas and Prevotella* [77], and is not more prevalent in root canal-treated teeth with lesions when compared to root canal-treated teeth with no lesions [132, 133]. Nevertheless *E. faecalis* is still relevant to endodontic research, and is the bacterium of choice to test the antibacterial activity of root canal sealers, irrigants, and disinfection procedures [134-137], as it is able to survive harsh conditions, and develop antimicrobial resistance [15].

2.2.2.1.1 Summary of *E. faecalis* virulence factors

- **Ace:** A collagen-binding MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules), it is expressed by the bacterium particularly under stress (the presence of serum, high temperature, and bile salts) [138, 139]. It was shown that the disruption of the *ace* gene impaired the conditional binding of *E. faecalis* to the extracellular matrix proteins (ECM) proteins [140]. Hubble *et al.* [141], tested the influence of Ace on adhesion of the bacterium to dentin as compared with the Ace-negative isogenic strain, Ace producing wild-type strain OG1RF adhered significantly more to dentinal surfaces.
• **AS:** Aggregation substance (AS) is a pheromone-responsive, plasmid-encoded bacterial adhesion that mediates efficient contact between donor and recipient bacterium [142], facilitating plasmid exchange. AS is proteinaceous that appears as a hair-like structure on the cell surface [143]. Its expression on the cell surface may be induced by serum [144]. AS was found to mediate binding to extracellular matrix (ECM) proteins, including collagen type I. Binding to collagen type I by bacteria may be of particular importance with respect to endodontic infections, since this is the main organic component of the dentin. Archimbaud et al. found that 45% of *E. faecalis* isolates from treated dental root canals had genes for AS [145].

• **EfaA:** EfaA (*Enterococcus faecalis* antigen A) is a solute binding-protein receptor for a manganese transport system in *E. faecalis*. The *efaA* gene was detected in all medical (blood, pus, urine, feces, hospital environment) and almost all food (milk, cheese, meat) isolates of *E. faecalis* [146]. The relatively low availability of manganese in dentin [147], may induce expression of *EfaA* in vivo [148].

• **Esp:** Enterococcal gene *esp*, encoding the high-molecular-weight surface protein Esp (*Enterococcal* surface protein), found rarely in stool isolates from healthy individuals [145]. *Esp* is associated with promotion of primary attachment and biofilm formation of *E. faecalis* on abiotic surfaces [149].

• **Gelatinase:** Gelatinase is an extracellular zinc-containing metalloproteinase from *E. faecalis*. It can hydrolyze gelatin, collagen, fibrinogen, casein, hemoglobin, insulin, certain *E. faecalis* sex-pheromone-related peptides, and some other bioactive peptides [150]. Singh et al. [151] found that gelatinase-producing *E. faecalis* strains are more lethal when compared with the deficient strains.

• **Hyaluronidase:** Hyaluronidase acts on hyaluronic acid and is mainly a degradative enzyme that is associated with tissue damage as the consequence of its activity. Hyaluronic acid as the substrate for hyaluronidase has been detected
in dentin [152]. Bacteria isolated from infected root canals associated with apical periodontitis produce hyaluronidase, and the hyaluronidase activity appears to be related to the degree (acute and subacute) of clinical symptoms [153]. Hyaluronidase ('the spreading factor') facilitates the spread of bacteria as well as their toxins through host tissues.

- **Cytolysin**: Cytolysin is a virulence factor consisting of two post-translationally modified peptides that synergistically kill human immune cells. Both peptides are made by CylM, a member of the LanM lanthipeptide synthetases [154]. The lytic factor precursors CylL_L (the long subunit) and CylL_S (the short subunit) are ribosomally synthesized and modified post-translationally by CylM [155]. The cytolysin-producing bacterium itself is protected from lysis by the cylI gene product, through unknown mechanisms [156].

- **Sex Pheromones**: Sex pheromones are chromosomally encoded, small, hydrophobic peptides, 7 or 8 amino acids long, which function as signaling peptides in *E. faecalis*. It was shown that antibiotic resistance and other virulence traits, like cytolysin production can be transferred between strains of *E. faecalis* by sex pheromone system [157].

![SEM image](image.png)

**Figure 2.3**: SEM (X 5k) of *E. faecalis* penetrating the dentinal tubules (arrows).
2.3 Identification techniques of root canal microbiota

Precise identification of microorganisms participating in the pathogenesis of apical periodontitis is important in order to understand the disease process and to develop more effective strategies for root canal therapy.

Culturing methods were considered as standard methods used in research [158, 159]. However they have drawbacks that can lead to underestimation of bacterial diversity [160]. A low number of bacterial cells may be sampled from root canals due to difficulty of sampling, and if the sensitivity of the method used for identification is low, many bacteria can pass unnoticed [114, 161]. Development of newer molecular methods, immunological assay, and microscopy techniques initiated a shift in the understanding of the root canal system microbiota [102].

The culturing method measures viable bacterial cells as colony-forming units while molecular methods measure nucleotide sequences and viable microorganisms are not required. Application of molecular methods for microbial detection has added several additional species as typical of the microbial flora of the infected root canals [102]. Numerous molecular techniques are available to identify root canal microbiota; DNA-DNA hybridization; PCR [162], and pyrosequencing [163] are the most commonly used techniques. DNA-DNA hybridization technology has the feature that microbial contaminants are not cultivated, nor is their DNA amplified [164], in addition to the reported advantages of molecular methods [162]. PCR is easier, faster and more sensitive in comparison with standard methods [110]. Currently pyrosequencing technique is gaining popularity as it is able to detect low-abundant microorganisms of endodontic microbiota [165].

Immunological methods are based on the specificity of antigen-antibody reaction. The enzyme-linked immunosorbent assay (ELISA) and the direct or indirect immunofluorescence tests are the most commonly used immunological methods for microbial identification [162]. They take no more than a few hours to identify a microbial species, can detect dead microorganisms, can be easily standardized, and have low cost.
However, they can detect only target species, they have low sensitivity, and their specificity is variable and depends on types of antibodies used [166, 167].

2.3.1 Microscopy techniques used to observe microbial biofilms in root canals

Several microscopy techniques are currently used to evaluate the sealer/dentin interface, including stereomicroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM) [168, 169]. Much of the early investigative work on biofilms relied heavily on the conventional SEM [92]. In this technique, samples must be fixed, dehydrated, and dried or frozen prior to observation, the dehydration process results in significant sample distortion and artifacts [170]. TEM has been also used for visualization of infected tubules in carious lesions and root canal infection on dentin specimens [171]. In this technique samples must be prepared prior to observation and therefore may develop artifacts [172].

CLSM has provided the ability to examine biofilms in situ without the limitations encountered with the SEM, albeit at lower magnifications [83], and has the advantage of providing detailed information about the presence and distribution of sealers or dental adhesives inside dentinal tubules in the total circumference of the root canal walls through the use of fluorescent marked sealers [169]. CLSM is now being used to determine the true architecture of plaque and the location of selected bacteria within the biofilm [173]. The trade off in resolution is more than offset by the ability to examine the biofilm matrix unaltered and intact [83]. The use of CLSM requires that the organisms in the biofilms be stained with fluorescent stains. These stains are designed to emit light at specific wavelengths and can be used to probe specific cellular functions. Using a suite of such stains allows the biofilm researcher to quantify all the cells and determine which ones are viable [83, 174].

2.3.1.1 Fluorescent stains

Confocal microscopy requires the use of fluorophores to visualize structures of interest within a specimen. To perform reliable measurements of the intensity of fluorescence, the stain should be specific, penetrate well into tissue sections, and fluorescence should be
Cell viability is an important parameter in tissue engineering and culture studies to evaluate long-term survival of cells [176]. The LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit allows bacterial cells to be distinguished according to cytoplasmic membrane permeability with the aid of a flow cytometer [33]. This kit uses a mixture of two nucleic acid stains - green-fluorescent SYTO® 9 dye and red-fluorescent propidium iodide - for viability determinations, and a calibrated suspension of microspheres for accurate sample volume measurements.

Stains displaying a signal in the ultra-violet (blue) spectral region, such as Rhodamine B, are known to produce a strong signal. However, such short wavelength excited fluorochromes are often insoluble in water and are prone to photo-bleaching [177].

For this reason, fluorescent dyes excited by longer wavelength energy and displaying emissions of longer wavelength light (specifically, green and red) are preferable. Commonly used long wavelength energy fluorescent stains such as fluorescein diacetate and ethidium bromide were explored. Ethidium bromide is a red fluorescent nucleic acid stain that permeates only cells with damaged cell membranes. Fluorescein diacetate penetrates all cells but is non-fluorescent until the green fluorescein moiety is freed by intracellular esterases. A disadvantage of fluorescein is that it is not retained in the cells for a prolonged amount of time; all analysis would have had to be performed within 15 min of staining.

2.4 Root canal sealers

An ideal root canal filling material should possess antimicrobial properties or at least discourage bacterial growth; therefore, proper filling of the root canal system is a fundamental step in the sealing of the canals to prevent reinfection of the canal, contamination of the periodontal tissues and subsequent periapical pathosis [178]. The standard endodontic filling material is composed of a core material, which is most of the time GP and sealer (binding agent). GP does not bind to root canal walls; a sealing
agent must be used to obtain a “hydraulic closure” of the canal system by filling the irregularities of the canal [179].

The ideal sealer should possess the following properties [180, 181]:

1) Tacky to provide good adhesion
2) Provide hermetic seal
3) Radiopaque
4) Mix easily
5) No shrinkage upon setting
6) No staining
7) Bacteriostatic
8) Set slowly
9) Insoluble in tissue fluids
10) Non-irritating
11) Soluble in a common solvent for retreatments
12) Not provoke an immune response
13) Neither mutagenic or carcinogenic


1. Zinc Oxide Eugenol based sealers: ZOE (zinc oxide eugenol)-based sealers have been considered the sealer of choice due to the antibacterial properties, ease of use, and sealing properties [179]. Zinc oxide exhibited antimicrobial properties against both Gram-positive and Gram-negative bacteria [184]. The sealing properties of ZOE sealers were inferior in comparison to other sealers due to the relatively high solubility of the ZOE sealer. ZOE showed cytotoxicity with different cell culture systems due to its eugenol content [185].
2. Epoxy resin based sealers: AH Plus® (Dentsply Maillefer, USA) is currently the most used epoxy resin based sealer that consists of a paste-paste system, delivered in two tubes in a double barrel syringe [186]. The epoxide paste contains radiopaque fillers and aerosol. The amine paste consists of three different types of amines, radiopaque fillers, and aerosol.

AH Plus has shown better properties when compared to other sealers [25]. It showed the lowest weight loss among the different root canal sealers in water and in artificial saliva with different pH values, independent of the solubility medium used [187]. Furthermore, AH Plus showed the greatest stability in solution, as compared to the conventional sealers [188].

3. Silicon based sealers: In 1984, silicon was first introduced as a root canal sealer. Silicones show comparatively little leakage, are virtually non-toxic, but display no antibacterial activity [189, 190]. GuttaFlow® (Coltène/Whaledent, Langenau, Germany) is a cold, fluid obturation system that combines sealer and GP in a single material, GuttaFlow powder with a particle size of less than 30 nm has been introduced into a silicone matrix (polydimethylsiloxane (PDMS)). GuttaFlow® is insoluble, biocompatible, and able to provide a thin film of sealer, and hence greater adhesion with dentin [191]. It showed poor wetting on the root dentin surface because of the presence of silicone, which possibly produces high surface tension forces, making the spreading of these materials more difficult in comparison to the other sealer [190].

4. Calcium hydroxide based sealers: Calcium hydroxide was introduced to the root canal in 1940, and later became popular for apexification, sealing perforations, management of resorption, and as a root canal sealer [192]. The availability of free hydroxyl ions in this sealer results with a high pH and hence provides an antimicrobial activity [193], and also activates alkaline phosphatase that plays an important role in hard tissue formation [194]. It diffuses through dentinal tubules and may communicate with the periodontal ligament space to arrest external root resorption and accelerate healing [195]. Calcium hydroxide–based sealers are not superior to other groups of sealers in their sealing or antibacterial
activity, and do not fulfill the criteria of the ideal sealer mentioned above with the main concern of its high solubility [196].

5. Glass ionomer sealers: Glass ionomer cement endodontic sealer is a biocompatible sealer [197], that chemically adheres to dental hard tissues. It exhibits no shrinkage upon setting, possesses superior adaptation to the canal walls and radiopacity as compared with Calcium hydroxide sealer [198], makes the root more resistant to vertical fracture [199], and showed acceptable clinical outcome [200].

The main concern regarding glass ionomer sealer is irretrievability if retreatment is needed; it was found that the sealer is retrievable with the use of solvent and ultrasonic instrumentation [201], but it is more time consuming in comparison with ZOE and epoxy resin sealers [202].

6. Calcium-silicate sealers: MTA (Mineral Trioxide Aggregate) (MTA; ProRoot MTA, DENTSPLY Tulsa Dental) is a tricalcium silicate cement that was first introduced as a perforation repair material [203], and recently as a root canal sealer. MTA has the ability to regenerate periodontal ligament and form cementum in the root canal space and accessory canals, thus closing the leeway spaces that can result in the treatment failure [204]. In addition, sealers based on MTA demonstrated apatite-like deposits in contact with physiological solutions and a biocompatibility similar to MTA [205]. However it is difficult to handle, and it has extended setting period [206]. AH Plus was found to bond better to the core obturation material and root dentin [207], and has the advantages of less shrinkage, high radio opacity, low solubility, better periapical repair and biocompatibility [208].

EndoSequence® BC Sealer™ (Brasseler, Savannah, GA, USA) is an example of calcium phosphate silicate-based bioceramic cement. It uses the moisture within the dentinal tubules after canal irrigation to initiate and complete the setting reaction. The pH of EndoSequence BC Sealer during the setting process is higher than 12, which increases its bactericidal properties [209]. EndoSequence sealer is non-toxic and biocompatible [210], and it has the potential to increase fracture toughness of endodontically treated roots.
[211]. On the other hand, retreatment could be a challenge with bioceramic sealers; it was found that conventional retreatment techniques are not able to fully retrieve the sealer from the canal [212]. In addition to that the sealer expands significantly upon setting and can be extruded from the root canal into the periapical tissue [213].

7. Methacrylate resin based sealers: Methacrylate resin based sealers were introduced to the clinical practice with the intent to bond the core material to the root dentin [214], and create a monoblock within the root canal space [215]. The intent of a root canal monoblock is to achieve a total bond, and hence a total seal of the canal space, with the purported advantages of simultaneously improving the seal and fracture resistance of the filled canals [216, 217]. The bond of these sealers to root dentin depends on the penetration of hydrophilic resin monomers (incorporated to facilitate resin invasion into the wet dentinal tubules [218]) into the conditioned dentin surface to create micromechanical interlocking between the dentin collagen and resin, forming a hybrid layer [219].

Four generations of methacrylate resin based sealers have been available commercially:

The first generation Hydron (Hydron Technologies, Inc, Pompano Beach, FL) appeared in the mid-1970s [220, 221]. The use of poly [2-hydroxyethyl methacrylate] (polyHEMA) as the major ingredient [222] rendered the sealer very hydrophilic. The sealer caused inflammatory reaction [223], absorption of the material [224], great leakage [225], as well as water sorption and swelling [226].

The second generation of bondable sealer is non-etching and hydrophilic in nature and does not require the adjunctive use of a dentin adhesive [227, 228]. EndoREZ (Ultradent Products Inc, South Jordan, UT) a dual- cured radiopaque hydrophilic methacrylate sealer is an example of the second generation.

The third generation is self-etching sealers that contain a self-etching primer and a dual-cured resin composite root canal sealer; FibreFill R.C.S. root canal sealant (Pentron Clinical Technologies, Wallingford, CT) and Resilon (Resilon Research LLC, Madison,
CT) are examples of the third generation.

To simplify bonding procedures, the fourth generation of methacrylate resin–based sealers (e.g., MetaSEAL, Parkell Inc; RealSeal SE, SybronEndo) were introduced, those sealers have further eliminated the separate etching/bonding step [229]. They possess additional benefits of reduced application steps and overall improvements in their user friendliness [230, 231].

Although some in vitro studies on the sealing ability [232, 233], root fracture resistance [234, 235], and removability of the sealers [236, 237] showed better potential over conventional nonbonding sealers, accomplishing the ideal goal of a monoblock in the root canal space with these materials is still a major challenge, and no clear benefit with the use of methacrylate resin–based sealers is achieved mainly due to the difficulty in achieving proper adhesion to intraradicular dentin [25, 238, 239].

2.5 Adhesion to intraradicular dentin

The modern concepts of adhesive dentistry started with the pioneering studies of Dr. Buonocore that revealed how etching with phosphoric acid could increase bond effectiveness, in terms of either bond strength or sealing ability [240]. Dental resin composites are composed of four major components: a polymeric matrix that is usually methacrylate based (commonly bisphenol A glycol dimethacrylate (BisGMA), triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), or ethoxylated bisphenol A glycol dimethacrylate (BisEMA), filler particles (commonly glass, quartz, or ceramic oxides), coupling agents between the filler and the matrix such as silanes, and an initiator/inhibitor polymerization system [241].

Two main adhesive systems are used to bond methacrylate resins to tooth dentin (Fig. 2.4); etch and rinse (total-etch) technique that removes the smear layer, and self-etch technique that is able to modify the smear layer to create a substrate for bonding [242]. In the etch and rinse technique, a gel of phosphoric acid is first applied as an etching step to demineralize and remove the superficial tooth layer, and is later rinsed away [243]. This process allows for the primer (which contains hydrophilic acrylic monomers that can re-
wet dentin to prevent collagen collapse) to be initially applied and then followed by the application of the adhesive resin. This allows the more hydrophobic adhesive to infiltrate into the created micro-pores, producing a matrix that mechanically interlocks the resin to the dentin or enamel, and produces what has become known as the hybrid layer [244]. In the etch and rinse technique, the primer and adhesive can be combined into a single bottle. Therefore, this adhesive can be applied in either a two or a three-step technique. Conversely, the self-etch technique eliminates the rinsing step by combining either etching and priming or etching, priming, and bonding agent together, which results in the etchant and priming agent remaining within the smear layer [245]. Several studies are suggesting that the hydrophilic nature of self-etch adhesives, due to the presence of acidic monomers, renders them more vulnerable to water sorption, which may result in greater susceptibility to degradation in comparison to conventional total-etch techniques [246].

The first adhesives were mainly based on BisGMA [247, 248], a very commonly used monomer [249], the bonding system was extremely hydrophobic (i.e., no compatibility with water) and thus, no adhesion was possible with the dentin tissue since it is an intrinsically wet substrate [59]. Later on the development and the incorporation of more hydrophilic monomers within the adhesive systems, such as 2-hydroxyethyl methacrylate (HEMA), allowed adhesives to bond also to dentin [250].

The concept of bonding within the root canal was initially adopted from restorative dentistry, as adhesive dentistry rapidly evolved and gained worldwide acceptance in the 1990s [251], further development of adhesive materials such as one-step self-etch primer systems [252, 253], dual- and self-cured materials, allowed for an easier adaptation of these materials for root canals therapy [217]. Methacrylate resin-based materials have been used in endodontic as root canal sealers, cements for posts and core restorations [254].

Despite major simplifications in dental adhesion, bonding to intraradicular dentin remains an unpredictable goal due to various clinical factors influencing the procedure; the presence and thickness of the endodontic smear layer, the use of irrigants or medicaments during the endodontic treatment, the use of eugenol-based materials or bleaching agents,
the possible incompatibility between different resin-based materials, geometric factors [59], and dentin moisture [39]. The unreliable bond between the root canal dentin and the resin material [25], made their use limited to post cementation [58, 59] and coronal restorations of endodontically treated teeth [60], thus their ability to maintain interfacial seal is still important to the success of the endodontic treatment.

2.5.1 Methacrylate resin luting cements for post cementation

Resin-based cements are superior to conventional cements for holding posts in their root canal post spaces [255], and are believed to reinforce the root [254]. Resin cements are classified according to their modes of curing as chemically-cured, light-cured and dual-cure. Chemically-cured luting cements are not easy to use as they have a very limited working time. Light-cured cements are difficult to cure at the apical part of the post space, even with the use of a light transmitting fiber post [256]. Hence, the dual-cure resin cements are commonly used for luting fiber post to intraradicular dentin [257].

In recent years, self-adhesive resin cements have gained popularity. This group of resin cements does not require etching and priming steps prior to application for cementation, as it combines the acidic and hydrophilic monomers in the cement composition. This unique composition performs demineralization and priming of dentin together with the luting function. This group of luting cements is probably easier to use as it eliminates several steps in the application that were required with the previous generation of luting cements, and therefore is claimed to be less technique sensitive [58].

On the other hand some researchers showed that the three-step etch-and-rinse adhesives remain the ‘gold standard’ and the simplification in the clinical application procedure results in loss of bonding effectiveness [32], the self-adhesive resin cements produced very low bond strengths to intraradicular dentin and among the different root regions; bond strength was the lowest in the apical third. The weaker etching potential of self-adhesive resin cements was thought to be the reason for the low bond strength and concluded that such cements [227, 258, 259].
2.6 Degradation of the methacrylate resin-dentin bond

The clinical longevity of resin materials depends on the structural integrity of the hybrid layer (resin-dentin interface) [260-262]. The resin-dentin interface can undergo degradation over time, allowing salivary and tissue fluid movement between the hybrid layer and dentin [32, 33]. A variety of chemical and physiological factors affect the durability of resin–dentin bonds [263]. In addition to the well-studied wear and mechanical function [264-266], salivary, bacterial, host immune, and dentinal enzymes in the oral cavity also contribute to the degradation of resin composites [44, 52, 267, 268].

2.6.1 Enzyme-catalyzed biodegradation

It was only in the early part of the 1990s that serious consideration was given to the possibility that enzymes, associated with saliva and oral tissues, may be involved in catalyzing chemical reactions which could degrade dental resin systems [269-271]. Enzymes in the oral cavity include; esterases, transferring enzymes such as catalases and oxidases, proteolytic enzymes such as proteinase, and others such as carbonic anhydrase.
Esterases are the most extensively studied class of enzymes against resin composites. This class of enzymes is derived from many different biological sources including salivary glands, inflammatory responses, microorganisms, and mononuclear phagocytic cells such as macrophages and monocytes that are commonly found in both normal and inflamed gingival tissues [272].

Polymers of resin-based composites are bound by unprotected ester linkages that are highly liable to cleavage by water [31, 273, 274]. Since the composition of human saliva is nearly 99% water, these materials are highly susceptible to hydrolytic degradation in vivo. It has been demonstrated that human saliva contains cholesterol esterase (CE) - and pseudocholine esterase (PCE)-like hydrolase activities [52], both esterases act synergistically to increase the biodegradation of the methacrylates BisGMA/TEGDMA composite resin materials [52, 274-276]. The hydrolysis of BisGMA-based composites yielded in the production of methacrylic acid (MA), and bis-hydroxy-propoxy-phenyl-propane (BisHPPP) (Fig 2.5) [276, 277].

The majority of studies assessing chemical breakdown of the resin matrix though have only been able to quantify overall byproduct release from the bulk composite resin restoration as a whole [275-278]. Since hydrolysis of HEMA generates the MA byproduct common to all methacrylate monomers [278], it has not been possible to isolate a degradation byproduct specifically generated from resin components of the marginal interface. BisGMA is a major constituent of the hybrid layer’s adhesive resin component, therefore quantifying the cumulative amount of BisHPPP release from a composite resin-restored tooth sample is currently the best available method of attaining some measure of implied chemical degradation occurring at the resin-dentin marginal interface [33]. BisHPPP contains no other hydrolysable ester bonds and is therefore a stable, single-source end product that accumulates in incubation media without undergoing any further hydrolytic reaction over time [277].
Figure 2.5: Esterase catalyzed hydrolysis of 2,2-Bis[4-2(2-hydroxy-3-methacryloxypropoxy) phenyl] propane (BisGMA) monomer (top) producing Bis-hydroxy-propoxy-phenyl-propane (BisHPPP) (left) degradation product and methacrylic acid (MA) (right), the unprotected ester bonds of BisGMA are marked by the red arrows. Adopted from Dr. Yoav Finer PhD thesis.

The hybrid layer is susceptible to degradation by MMPs [279]. MMPs are present in human saliva and are secreted by a variety of connective tissues and pro-inflammatory cells including fibroblasts, endothelial cells, macrophages, neutrophils, lymphocytes, and odontoblasts [279, 280]. To date many MMPs have been isolated and identified in dentin including stromelysin-1 (MMP-3) [281], neutrophil collagenase (MMP-8) [282], and gelatinases A and B (MMP-2 and MMP-9, respectively) [283]. MMPs are involved in many physiological processes including morphogenesis, angiogenesis, and dentinogenesis. However, these endopeptidases are also capable of degrading components of the extracellular matrix (ECM) including fibrillar and non-fibrillar collagens, fibronectin, laminin, and basement membrane glycoproteins [267]. Several MMPs including MMP-2, MMP-8, MMP-9, and MMP-20 participate in caries lesions by hydrolyzing collagen fibrils at specific amino acid sites [284, 285]. Consequently, the dentin–resin interface becomes compromised [280]. However, for this process to occur, these MMP enzymes must be activated.
MMPs require metal ions such as calcium or zinc to bind to the active site for their catalytic activation through a so-called cysteine switch [267]. Recent studies are suggesting that MMPs from saliva and those that are normally bound to mineralized collagen fibrils may become catalytically activated under acidic conditions [267, 280, 284]. A low pH alters the conformation of the protein, and induces the cysteine switch that activates the catalytic activity [286]. Therefore MMPs may become activated during acid etching, or upon bacterial acid production at the tooth and restoration interface [280, 286]. With large quantities of water present in the dentin and saliva, activated MMPs are able to hydrolyze the collagen, and compromise the stability of the resin–dentin interface [284].

Dentin is composed of an inorganic component (50 vol%), organic component (30 vol%) and water (20 vol%) [62]. The inorganic component is mainly hydroxyapatite minerals. Collagen is the major protein found in dentin, it constitutes ~90% of the organic matrix, most of which is type I collagen [62]. Dentinal collagen serves a variety of structural roles including shaping and organizing extracellular matrices [287], providing a scaffold for minerals formation and deposition [288], cells adhesion and differentiation [289], and preserving the structural, mechanical and functional integrity of the dentin [51]. Dentin damage might occur during the root canal treatment due to the strong and caustic root canal irrigants and medicaments used for cleaning of the root canals [290, 291], degradation/resorption during disease process [292, 293], and post-treatment degradation of dentin surface collagen by bacteria and MMPs [63, 141]. The amino acid sequence of collagen type-I contains glycines, and rich in hydroxyproline and proline [294]. Hydroxyproline is a collagen degradation product; its presence in the hydrolysate indicates the tissue-collagen concentration [295, 296], the stability, and resistance of collagen fibers to degradation by proteases [297, 298].

Bacterial infection caused by coronal leakage is a significant contributor to endodontic failure [64]. Bacterial protease collagenolytic enzymes have been linked to many human diseases [50], and may contribute to the degradation of collagen fibrils and deterioration of structural integrity in root dentin with time [63]. The ability of the purified gelatinase from *E. faecalis* to degrade soluble and insoluble collagens provides further indication of
the ability of the bacteria to degrade the organic material of the demineralized dentin [150]. This protease activity could possibly explain part of its role in root canal infections by facilitating the migration of *E. faecalis* and other pathogenic species from the root canal into the periapical lesion and may explain the role of Enterococci in apical periodontitis [15]. Bacterial proteases include some metalloproteases that cleave collagen in a similar manner to human MMPs [299]; MMPs have been widely studied for their important role in mammal development and human diseases [300, 301]. MMP-1, -2, -8, and -9 have been shown to be involved in degrading dentinal collagen [34, 282, 302]. Therefore, it could be hypothesized that bacterial proteases have similar effect and degrade dentinal collagen.

### 2.6.2 Interfacial marginal integrity

Assessing the interfacial (sealer-dentin) integrity gives an indication of the quality and the amount of porosities created by different root canal filling materials and techniques that could adversely affect the outcome of root canal treatment [42]. In addition to salivary enzyme infiltration, a compromised interface allows for the bacterial proliferation into the margins between tooth and restoration [33].

*In vitro* microleakage studies have been the most widely used approach to investigate the root filling quality, due to their relative ease, reproducibility and cost. Variations of these models include using dyes and tracers [303]; saliva [56, 304, 305]; fluid filtration models [306]; bacteria [307]; endotoxins [308]; and glucose filtration [309].

*In vivo* research encompasses both animal studies and clinical studies. Investigations have been performed using dog models, where endodontically treated teeth were challenged through bacterial ingress and subsequent histological analysis evaluated the periradicular response to different tested materials [178, 310]. Unfortunately, the correlation between these results to clinical outcomes is not straightforward, as shown by Pitt Ford [311], where he showed a lack of correlation between dye penetration and periapical tissue response in dogs’ teeth.
Research that primarily relies on the penetration of a marker is based upon relatively simple and predictable mechanisms [312]. Conversely, the clinical outcome predictors associated with endodontic disease are far more complex and governed by intricate biological systems [313]. In order to embark on in vivo research there are significant financial and ethical obstacles that must be met. While evaluating the efficacy of a novel application of biomaterials, it would be prudent to first embark on relevant in vitro studies that mimic clinical conditions prior to considering in vivo research.

Recent studies, have provided an alternative method to analyze bacterial colonization at interfaces between teeth and biomaterials using non-invasive methods [33, 42, 314]. Roth et al. [42] investigated the biofilm proliferation within the root canal sealer-dentin interface using CBBF, which allows for the continuously controlled flow of media thereby simulating in-vivo pathogenic oral conditions. Where interfacial disruptions and marginal gaps were present, bacterial biofilms colonized these sites and infected dentinal tubules. The adaptation and amalgamation of the systems presented in these studies [42, 314] provides a model that is biologically relevant and simulates in vivo settings.

The presence of bacteria between the tooth and restoration is a major challenge and a potential cause of postoperative sensitivity, secondary caries, pulp inflammation, and necrosis [44, 315, 316]. Salivary proteins are known to have a particularly high affinity for polymeric materials [317, 318], which could account for the increased growth of bacterial biofilm found along composite resin restorative margins [315]. Interactions taking place at the surface of polymeric resin materials appear to modulate certain bacterial characteristics relating to vitality at the cellular level [319, 320]. Exposure to resin-based biodegradation byproducts impacts the gene expression and growth of some oral bacteria [321-323].

Possible mechanisms for microbial degradation of polymeric composites include: direct attack of the resin by acids or enzymes, blistering due to gas evolution, enhanced cracking due to calcareous deposits and gas evolution, and polymer destabilization by concentrated chlorides and sulfides [263, 324]. Any attack may result in loss of strength due to fracture, disbonding, or delamination, and ultimate failure [263]. Bacteria
colonized all resins, fibers, and composites, preferential bacterial colonization of surface anomalies was consistently observed [263].

To increase the longevity of current resin composites, a means for effectively reducing and controlling the growth of cariogenic bacteria may be required to overcome the adverse effect of such degradation by-products [241, 249].

Most of the radicular dentinal tubules run perpendicular to the pulp and the periphery in the root canal. Their size and number differ along the root, with diameters ranging from 1 to 3 μm and density from 4900 to 90,000 tubules per square millimeter [325]. The diameter of the dentinal tubules is large enough to allow penetration by different bacterial species [326, 327], whose size may not exceed 0.3 μm. Bacterial penetration into dentinal tubules can extend to different depths. The degree of bacterial invasion depends on the type of bacterial species, the time of incubation [328, 329], and the age of the patient; bacterial infection of dentinal tubules occurs to a lesser extent in older patients [330], probably due to an increase in mineral content within the dentinal tubules which in turn results in their occlusion.

### 2.7 Summary

Root canal infections are caused by microbial proliferation into the root canal system. The aim of root canal treatment is to eradicate the infection and seal the canals properly to prevent further bacterial colonization. The interface between root canal sealers, post cement, coronal restorations and dentin degrades with time allowing for bacterial proliferation, reinfection of the root canal system, and treatment failure. Salivary and bacterial enzymes might accelerate the interfacial degradation by degrading both the dentinal side of the interface and the restoration side. Esterases are a group of enzymes that were detected in saliva and as a virulent factor secreted by some bacteria, and found to catalyze the degradation of the resin-dentin interface [33, 43, 44]. Up to our knowledge to date no research was done to explore the potential effect of these enzymes on the stability of the root canal sealer-dentin interface and on the physical properties of the root canal sealers. Another important group of enzymes is proteases, specifically collagenases
and geltinases that were found in human coronal and radicular dentin, saliva, and produced by many types of bacteria. The collagenolytic activity from dentin and saliva has the ability to degrade the dentinal collagen matrix. Bacterial collagenases and their effect on the dentinal collagen degradation are understudied and many undiscovered bacterial collagenolytic proteases need further investigations. The exploration of these proteases could provide further understanding of bacterial pathogenicity in root canal infections and will be helpful for the development of therapies for the diseases caused by relevant pathogenic bacteria. The current study is investigating the effect of the salivary-like esterases and bacterial esterases and collagenolytic-like activities on the biostability of the root canal-sealer interface by using recent identifications techniques.
Chapter 3

Interfacial Sealer-Dentin Integrity is affected by Sealer Type, Incubation Period, and Condition

*Muna Q. Marashdeh*¹,², *Celine Lévesque*¹, *Shimon Friedman*¹, *Yoav Finer*¹,²

¹Faculty of Dentistry, ²Institute of Biomaterials and Biomedical Engineering

University of Toronto

Abstract:

**Background:** This study assessed the biostability of different root canal sealer-dentin interfaces under simulated *in vivo* pathogenic oral conditions. **Methods:** Canals of human anterior tooth-roots were prepared and filled with gutta-percha and either: Self-cured resin composite (RC) (Bisfil® 2B) with either self-etch (SE) (EasyBond), or total-etch (TE) (Scotchbond®) methacrylate-based adhesives, epoxy resin (ER) (AH Plus®), or Bioceramic (BC) (EndoSequence® BC Sealer™). Specimens were pre-incubated in simulated human salivary esterase (SHSE) or phosphate buffered saline (PBS) for up to 360 days, followed by 3-day incubation in a chemostat cultivating steady-state *Enterococcus faecalis* biofilm. Live/dead staining of roots and confocal laser scanning microscopy were used to assess the depth of interfacial bacterial biofilm proliferation.

**Results:** Initial depth of interfacial bacterial biofilm proliferation was not significantly different between groups (p>0.05). All groups showed significant deeper proliferation with increased aging period regardless of the pre-incubation condition (p<0.05). SHSE incubation caused an increase in interfacial biofilm depth for TE for all aging periods, SE after 30-days, and BC after 180-days (P<0.05). While ER was not affected by SHSE pre-incubation. **Conclusions:** Interfacial bacterial biofilm proliferation was dependent on sealer type, pre-incubation period and condition. SHSE accelerated the interfacial degradation of methacrylate and BC, and this was indicated by increased interfacial biofilm proliferation and higher live/dead ratio.
Key words: sealer-dentin interface, biofilm proliferation, salivary enzymes, *Enterococcus faecalis*. 
Introduction

Kakehashi et al. [1] showed that pulpal and endodontic problems are primarily related to microbial contamination of the root canal system. The dominant pattern of bacterial colonization of the root canal system is biofilms [10-12]. It has been found that microbes still remain even after thorough chemomechanical preparation of the root canal system, and could proliferate to reach levels similar to that before treatment [7, 70]. *Enterococcus faecalis* is a microorganism commonly detected in persistent endodontic infections due to its many virulence factors and ability to survive under harsh conditions [15, 68, 117, 122]. To eliminate residual bacteria and/or prevent their ingress and proliferation, the canal is filled after the disinfection procedure [17]. The standard root filling is a combination of core material and sealer cement. An ideal endodontic sealer should provide antimicrobial activity, an excellent seal when set, dimensional stability, tolerance to tissue fluids, and mechanical strength [331, 332]. Currently, epoxy resin based sealers are considered the gold standard of all sealers as they possess good physical properties, and ensure adequate biological performance; however, low bond strengths are reported for the epoxy resin sealers to core material [207, 333]. Methacrylate resin based dual-cure endodontic sealers that are presumed to bond to the root canal walls were introduced to the clinical practice [334]. Resin adhesive systems are designed to bond resin composites to tooth structure and improve the sealing capacity of the root canal filling. Two main adhesive systems are currently available: etch and rinse technique that is designed to remove the smear layer and self-etch technique that modify the smear layer [335]. The resin-dentin interface undergoes degradation over time, allowing salivary and tissue fluid movement between the hybrid layer and dentin [33, 42]. This degradation process is further accelerated by simulated human salivary esterases (SHSE) [33, 43] and cariogenic bacteria [44]. Methacrylate resins have the potential to be used as a root canal sealers, but their use nowadays in root canal is mainly for post cementation [58, 59], and restoration of endodontically treated teeth [60]. BC is a newly introduced calcium phosphate silicate-based cement that uses the water in dentin to initiate and complete its setting reaction [336], presume to form hydroxyapatite, and ultimately create a chemical bond between dentinal wall and the sealer [337, 338]. On the basis of the above introduction, it would be beneficial to the oral health community to assess the biostability
and interfacial integrity of different endodontic sealer-root dentin interfaces over time. The purpose of this study was to investigate the effect of SHSE exposure over time on the marginal breakdown at the sealer-root dentin interface of four different groups; methacrylate-resin composite (RC) (Bisfil 2B, Bisco, USA) with either self-etch (SE) adhesive (EasyBond), or total-etch (TE) adhesive, ER (AH plus, Dentsply-Tulsa, USA), or BC (EndoSequence BC Sealer, Brasseler USA, Savannah, GA). The depth of interfacial *E. faecalis* biofilm proliferation and interfacial bacterial live/dead ratio under these conditions was used to assess marginal integrity.

**Methodology**

**Specimen’s preparation:** Human anterior caries-free teeth were collected and kept stored at -20 °C in distilled water (University of Toronto ethical approval # 28214) until ready for use. Teeth were inspected for cracks under the operating microscope. The crowns of the teeth were cut; canals were prepared using parallel drills (ParaPost®, Coltene, Ohio, USA) gradually from 0.9-1.5 mm, and inspected for cracks. After drilling, teeth were autoclaved [339]; autoclave had no effect on the marginal seal and dentin properties [340]. Endodontic treatment procedures leading to root filling were performed in a biohazard safety cabinet. Canals were irrigated with 5 mL of 5.25% NaOCl, 5 mL of 17% EDTA, followed by a final flush with 10 mL of sterile distilled water, and then dried with sterile paper points. Teeth were randomly assigned to one of four experimental groups: RC with TE (Scotchbond™), or SE (EasyBond), ER (AH plus), or BC (EndoSequence), all groups were filled with gutta-percha points as a core. The filled roots were stored for 72 h in a 100% humid environment at 37°C to allow the sealers to completely set, then 3 mm root segments were obtained from the coronal part of each root using a slow-speed, water-cooled rotary diamond disc. The apical and coronal surfaces of the segments were polished with silicon carbide grinding papers of 600-1200-grit.

**Specimens pre-incubation:** From each root one sample (segment) only was obtained. Three experimental samples were assigned for each group, and three regions of interest (ROI) from each sample were examined under the microscope (3x3= 9 readings/group).
The samples were either non-incubated (control), or incubated in either SHSE, or PBS (37°C, pH 7.0). Specimens in each experimental group were assigned to a time 0 control (non-incubation) or an incubation period of 30, 180, and 360 days. SHSE was prepared by mixing pseudocholine esterase (PCE) and cholesterol esterase (CE) solutions. Incubation solutions were replenished throughout the pre-incubation period to keep the esterase activities in levels corresponding to activities present in human saliva using a method described previously by Serkies et al. [53], maintaining 16 Units/mL and 0.01 Units/mL for CE and PCE activity, respectively.

Biofilm cultivation: Following the assigned pre-incubation period, specimens from each incubation period were suspended in a Chemostat Based Biofilm Fermenter (CBBF) to cultivate steady-state biofilms of *E. faecalis* ATCC 29212, for 3 days in Brain heart infusion (BHI) (pH=7.0, 37°C) [33, 42]. An overnight culture of *E. faecalis* ATCC 29212 in BHI was used to inoculate the CBBF, before pumping a fresh medium (1/2 BHI supplemented with 20% glucose w/v and 40 mM phosphate citrate buffer) into the vessel at a flow rate = 1.6 mL/min, within the range of human salivary flow rate [341]. To provide a baseline for background fluorescence, morphological baseline and bacterial presence for the subsequent microscopic examination, one specimen from each sealer group was subjected to each one of the following procedures:

A. No pre-incubation, incubation in the CBBF without bacterial cells, and staining.
B. No pre-incubation, incubation in the CBBF with inoculation, and staining.
C. No pre-incubation, no incubation in the CBBF, and staining.

Outcome assessment: Specimens were removed aseptically from the CBBF, gently rinsed with distilled H2O once, then stained using LIVE/DEAD® stain (*BacLight™* Bacterial Viability Kit, Invitrogen, USA). Stained specimens were assessed individually for marginal interface morphology, and bacterial biofilm proliferation, penetration and viability by using Confocal Laser Scanning Microscope (CLSM), (Zeiss LSM710 Two-photon and confocal microscope, MaRS Discovery District-South Tower, Toronto). The sealer-dentin interface was identified, and 3 cardinal points were scanned using a 20X (water-immersion)/NA 1.0 objective lens. Z-stack sequential images were
collected at 1-μm intervals between images. CLSM Z-stack images were processed by IMARIS (Bitplane AG, Zürich, Switzerland). This software can remove background fluorescence and allow for quantification of bacterial cells [342].

Analysis: After testing for normal distribution, three way ANOVA and Scheffe post hoc analyses (p<0.05) were used to determine the effect of sealer type, pre-incubation media, and time on the depth of bacterial proliferation identified within the sealer-dentin interface and the bacteria live/dead ratio.

Results

Bacterial biofilm proliferation depth: Figure 3.1 shows representative CLSM Z-stack images processed by IMARIS for one of the regions of interests of ER sample. Biofilms proliferation depths for the all-material groups are depicted in Figure 3.2. There was no significant difference in the initial depth of interfacial bacterial proliferation for the four groups (p>0.05). After 360 days all groups showed significant deeper proliferation regardless of the pre-incubation condition compared to baseline (p<0.05). Following 30 days of pre-incubation, bacterial proliferation for the resin composite groups was significantly deeper (p<0.001) for SHSE pre-incubated groups compared to controls. This trend was consistent for TE only for all incubation periods (p<0.05), ER showed no significant difference between pre-incubation in SHSE or saline for all aging periods. The interface of BC was unclear under the microscope due to the material expansion at 30-days, but 180 days pre-incubation group showed a significant increase in bacterial proliferation for SHSE vs. PBS (p<0.05).

Live-dead ratio (Fig. 3.3): At zero pre-incubation time the live dead ratio was significantly lower for BC group (0.72±0.27), followed by ER (2.86±0.43), then TE (4.92±1.04), and the least ratio was for SE (6.69±2.1) groups. The ratio of all groups decreased after 30 days of pre-incubation, and started to increase again with time regardless of the incubation media. Pre-incubation with SHSE increased the live/dead ratio significantly for TE-30, SE-30, TE-180, ER-180, BC-180, SE-360, and BC-360 days compared to PBS saline (p<0.05). After 360-days BC sealer still showed the lowest
live/dead ratio of all groups.

**Discussion**

This study confirmed that none of the tested materials provided hermetic initial seal of the interface, and all interfaces showed increased degree of degradation with time, as indicated by bacterial biofilm proliferation. The level of interfacial degradation was dependent on sealer type, pre-incubation period and condition. SHSE incubation accelerated interfacial degradation, of BC, TE, and SE at some time points, and increased the live/ dead ratio for all groups at different time points.

ER was selected as it is considered the gold standard in comparative studies of root canal sealers due to its favorable physical, biological and antimicrobial properties [23-25, 343]. BC sealer is a newly introduced sealer that showed tolerance to moisture and small amounts of blood, good antimicrobial properties, sealing, and mechanical properties [35, 344]. The third material, RC is a methacrylate resin polymer that could bond to dentin but is vulnerable to hydrolysis by salivary [52], and bacterial [44] esterase and increase the degradation of the resin-dentin interface at a greater rate [33, 43]. Methacrylate resins have the potential to be used as a root canal sealer, but it is mainly used nowadays in root canal for post cementation [345], and restoration of endodontically treated teeth [346, 347]. Either TE or SE adhesive systems are used to bond RC to tooth structure. In the absence of proper coronal seal, saliva and microbes can cross the root canal affecting the treatment success [348]. Therefore, it is imperative to investigate the effect of the sealer-dentin interface in an environment where critical salivary enzymes are present and explore any detrimental effect on their performance.

Saliva has been shown to contain both CE and PCE-like esterases activities [52]. SHSE is a mixture of CE and PCE that was chosen for its potential to affect the sealer-dentin interfacial stability. Both esterases acted synergistically to increase the biodegradation of methacrylate resin dental composites and adhesives [52], decrease their bond to dentin [43, 53], and increase the bacterial biofilm proliferation between the adhesive-dentin interfaces [33], and affected some of the physical properties (microhardness, weight loss, and dimensional change) of the bioceramic sealer and resin composite (Chapter 4).
Samples were first aged (pre-incubated) individually in sterile glass vials containing either PBS or SHSE for up to 360 days. SHSE is a mixture of CE and PCE activities, mimicking salivary esterase activities that were shown to affect the restoration-tooth interfacial stability [33, 43, 52, 53].

Following the pre-incubation period, specimens were incubated in CBBF using a previously described model that allowed for continuous controlled flow of BHI simulating pathogenic oral conditions to cultivate *E. faecalis* biofilm [33, 42]; a facultative anaerobic cocci commonly detected in asymptomatic, persistent endodontic infections [15] that was used as a biological marker to assess the interfacial stability. The survival of *E. faecalis* after endodontic therapy has been attributed to many virulence factors [15, 68, 117, 122], one of the most important factors is the ability to form biofilm [13]. The rationale for using separate phases of first, exposure to SHSE and only then incubation with *E. faecalis* is based on the need to prevent interactions between the proteins in SHSE and the bacteria, and also based on practical limitation of long-term biofilm growth under *in vitro* conditions [33].

CLSM is a non-destructive technique that has provided the ability to examine biofilms *in situ* without the limitations encountered with 2-D destructive and static methods such as scanning electron microscopy [33, 83], CLSM is used to determine the true architecture of plaque and the location of selected bacteria within the biofilm [173]. The use of CLSM requires that the organisms in the biofilms be stained with fluorescent stains. Using a suite of such stains allows the biofilm researcher to quantify all the cells and determine which ones are viable [83, 174], and this could be used as an indication of the antimicrobial activity of the materials [349].

The depth of bacterial biofilm proliferation varied between the four material groups at different time points and pre-incubation media. At time 0 all materials showed comparable sealing ability that deteriorated with time to end up after 360 days having the best sealing ability for BC and ER in SHSE, and for BC in PBS. ER was reported to be more stable in solutions and salivary enzymes compared to other sealers [187, 188]. The current study corroborated these findings and confirmed that ER groups were not affected
by the enzyme exposure; this could have been due to the material’s hydrophobicity and lack of ester bonds that are susceptible to hydrolysis [188]. SHSE increased the depth of bacterial biofilm proliferation after 180 days of exposure for TE and BC compared to PBS incubation. BC has been shown to expand during and after setting [350], the current study showed that the surrounding media greatly affect the expansion process [350]; incubation in SHSE decreased BC expansion, and increased its solubility (Chapter 4). This could explain the finding of this study; the continued expansion of the material after setting could have resulted with the difficulties encountered with imaging the 30-days group, that later resolved due to the material’s apparent solubility (Chapter 4). After 360 days of pre-incubation in saline BC was the least prone to bacterial proliferation probably due to the material expansion, and its interfacial sealing capacity that does not rely on degradable monomers [35]. Methacrylate resin-dentin interfaces were the most susceptible to interfacial biofilm bacterial proliferation; SHSE incubation increased this susceptibility probably due to hydrolysis of the ester-links within the resin materials [52], and increase the shrinkage of the material (Chapter 4) resulting in interfacial degradation and bacterial biofilm proliferation [33]. Despite being more hydrophilic and therefore it is more susceptible to undergo hydrolytic degradation [44], and reduction in mechanical properties [43], SE group showed better performance when compared to TE at 360-day in SHSE and this result was in agreement with Serkies et al. [53], who showed superior strength and preservation of interfacial integrity of SE. The better performance of SE could be due to its better suitability to be used in the complex root canal geometry than TE [229], since it does not require a separate etch and rinse step, and due to materials’ inherent bulk differences in chemical composition [44].

Comparing the four test materials, BC maintained the highest antimicrobial activity, while the lowest activity was for the methacrylate resin groups that showed previously no antimicrobial advantage over the other sealers [351]. Increasing the number of live bacteria in some of SHSE pre-incubated groups could be explained by possible interactions between the protein and the bacteria [352], as well as the increase in interfacial gap with time [33, 42]. ER and BC showed reduction in their antimicrobial activity with time, and this result corroborate other studies that showed that most sealers
lose their antibacterial activity over time [351, 353, 354]. The reduction in the antimicrobial activity could be due to its dissolution in aqueous media that may also eventually affect its sealing capacity (Chapter 4).

**Conclusions:** Within the limitations of this *in vitro* study, BC sealer showed the best sealing ability and antimicrobial activity when compared to other sealers after 360 days of aging in saline, while its sealing ability was comparable to ER after 360 days of SHSE exposure. SHSE exposure increased bacterial biofilm proliferation depth and live/dead ratio, and therefore could compromise the result of the root canal treatment. This highlights the importance of using incubation media with enzymatic activities similarly to those that exist in human saliva rather than the use of buffers or water as incubation media for the assessment of aging on the material sealing ability.
**Figure 3.1:** Representative confocal images (212.1 x 212.1 μm), for ER, 30 days PBS pre-incubated sample, with bacterial proliferation depth of 13 μm; showing the interface (yellow line) between Root canal filling (F), and dentin (D), at depth 0 μm (a), 5 μm (b) showing bacterial biofilm (white arrows), and 13 μm (c) where no more bacterial biofilm can be detected.
Figure 3.2: Biofilm proliferation (μm) for the test groups (SE, TE, ER, and BC) after being exposed either to A) PBS or B) SHSE for different time periods (0, 30, 180, and 360 days). Data are shown as mean ± SD. Initially the four materials showed a comparable seal that deteriorated with time. For BC groups the interface was not clear under the microscope due to the material expansion after 30 days of incubation. BC had the shallowest bacterial proliferation after 360 days in PBS, while the depth was comparable to ER after pre-incubation in SHSE and was less than SE and TE. Different letters represent statistical significance between materials at the different pre-incubation periods and media.
Figure 3.3: Schematic diagram showing the interfacial bacterial cell live/dead ratio for the test groups (SE, TE, ER, and BC) after being exposed either to A) PBS or B) SHSE for different time periods (0, 30, 180, and 360 days). Data are shown as mean ± SD. Initially BC had the least live/dead ratio (highest antimicrobial activity) followed by ER, and the highest ratio was for TE and SE. The live dead ratio decreased for all the materials after 30 days of pre-incubation in both SHSE and PBS, and started to increase again with longer periods of SHSE and PBS exposure. BC maintained the highest antimicrobial activity after 360 days of pre-incubation. Different letters represent statistical significance between materials at the different pre-incubation periods and media.
Chapter 4

The Effect of Simulated Human Salivary Esterases on the Physical Properties of Root Canal Sealers

Muna Q. Marashdeh¹,², Celine Lévesque¹, Shimon Friedman¹, Yoav Finer¹,²

¹Faculty of Dentistry, ²Institute of Biomaterials and Biomedical Engineering
University of Toronto

Abstract:

Background: The purpose of this study was to assess the effect of simulated human salivary esterases (SHSE), known to degrade dental methacrylate resins, on the physical properties of root canal sealers. Methods: Surface microhardness, compressive strength, dimensional change (DC %), and solubility/ weight loss (WL %) of bioceramic sealer (BC), epoxy resin sealer (ER), and resin composite (RC) were tested after exposure to SHSE or Phosphate Buffered Saline (PBS) for up to 4 weeks. Results: Microhardness of ER remained stable throughout the incubation period in both media. Microhardness of BC went up, and that of RC went down with time compared to the base line and regardless of the incubation media. SHSE reduced microhardness compared to PBS incubation of BC (28.0±4.8 vs. 38.1±7.9 KHN) at 7 days, RC (55.6±7.1 vs. 66.3±6.5 KHN) at 7, and 28 days (52.3±9.2 vs. 62.6±8.5 KHN). SHSE had no significant effect on the compressive strength of the three materials when compared to PBS. BC expanded with time and the expansion was less after 7 days of SHSE exposure compared to PBS (0.026±0.01 vs. 0.049±0.007 DC%), ER and RC showed shrinkage with time that was not affected by media type for ER and was higher for RC after 7 days of incubation in SHSE (0.5±0.07%) compared to PBS (0.38±0.08%). BC had the highest solubility among the three materials that increased significantly after 28 days of incubation in SHSE compared to PBS (2.40±0.2 vs. 2.96±0.19 WL%), followed by RC that showed significant increase in solubility after 7 days incubation in SHSE compared to PBS (0.54±0.09 vs. 0.80±0.1), ER was the least soluble material. Conclusions: SHSE affected
some of the physical properties of BC and RC. ER sealer showed the highest stability in both PBS and SHSE.

**Key words:** root canal sealer, physical properties, bioceramic sealer, epoxy resin sealer, resin composite, salivary esterases.
**Introduction**

The standard endodontic filling is a combination of a sealer with a central core material. The core acts as a piston on the flowable sealer that adapts to the root canal’s walls. An ideal endodontic sealer should provide antimicrobial activity, an excellent seal when set, dimensional stability, tolerance to tissue fluids, and mechanical strength [331, 332]. The chemical and physical properties of root canal sealers such as microhardness, compressive strength, solubility, and dimensional change might have an effect on the quality of root canal filling and consequently on the outcome of the endodontic treatment [21, 22].

Sealers come into contact with the oral physiological solutions such as blood and/or saliva [54-57]. Moisture can have a crucial effect on the setting process of sealers [41], and consequently on their sealing ability [228, 355]. Human saliva contains several enzymes; esterases; catalases; oxidases; proteinase, and others [272, 335]. Esterases are a group of enzymes that can catalyze the hydrolysis of ester bonds. Human saliva contains cholesterol esterase (CE) and pseudocholine esterase (PCE)-like hydrolase activities [52]. Both CE and PCE act synergistically to increase the biodegradation of methacrylate dental resins [52], decrease their bond to dentin [43, 53], and increase the bacterial biofilm proliferation between the resin-dentin interfaces [33].

Currently various endodontic sealers are available commercially; AH Plus is an epoxy-based resin sealer (ER) that is widely used among the endodontic community due to its favorable physical and biological properties [23-25]. However it lacks the ability to bond to the core material and the tooth structure. New sealers are constantly being developed in attempts to provide better properties by improving the bonding to the tooth, and/or to improve biocompatibility and/or improve antibacterial properties. Methacrylate resin-based root canal sealers were invented with the intent to overcome the drawbacks of the traditional sealers by bonding to both root canal dentin as well as the core material [26, 27, 215], however their apparent improvement did not result with the anticipated outcome [25], and the use of methacrylate resins nowadays in root canal is mainly limited to post cementation [58, 59], and restorations of endodontically treated teeth [60]. Bioceramic-
based sealers (BC) are newly developed sealers that were developed following the positive outcome of bioceramic technology in medicine and dentistry [23, 35]. This material is biocompatible [36], contains calcium phosphate, which improves the interaction of these materials with dentin (the mineral infiltration zone) [37, 38], requires moisture to set [39], while contamination with blood and saliva might adversely affect its performance [40, 41].

The purpose of this study was to investigate the effect of incubation in SHSE on some of the critical physical properties related to the performance of endodontic sealers after observing deterioration in the sealing ability of the sealers when aged in SHSE in a previous study (Chapter 3), including microhardness, compressive strength, dimensional stability and solubility of three dental materials, BC (EndoSequence BC Sealer, Brasseler USA, Savannah, GA), ER (AH plus, Dentsply-Tulsa, USA), and methacrylate-resin composite (RC) (Bisfil 2B, Bisco, USA).

**Methodology:**

Technological tests that have been used in this study followed the methods recommended by standards organizations; the American National Standards Institute/American Dental Association (ANSI/ADA 2000) to assess the physical properties of endodontic filling materials and dental water-based cements [356, 357], and the International Organization for Standardization (ISO 6876, 2012) for dental root canal sealing materials [358].

**Microhardness test:** Specimens (n=5/material in each group) with an inner diameter of 10±0.1 mm and a thickness of 2±0.1 mm were used. For BC sealer that requires moisture for setting, plaster of Paris molds containing a cavity of 10-mm diameter and 1-mm height were used [16]. The assembly of ring molds and mixed material were placed in an incubator with 95-100% humidity at 37°C for 72 h. Surfaces of the specimens were wet polished using hand pressure and silicon carbide-based sandpapers of varying particle size (600-1200-grit; 3M, St Paul, MN, USA). The specimens were cleaned using distilled water to remove surface debris, and were dried gently by air spray [359-361]. Afterward, the samples in each group were randomly divided into five subgroups (n=5/group): A) no
incubation, B) 7 days in PBS, or C) SHSE, D) 28 days in PBS, or E) SHSE. SHSE was prepared according to a method described by Serkies et al. [53]. SHSE solution were changed to maintain the activity at 16 Units/ml and 0.01 Units/ml for CE and PCE, respectively. Samples were removed at the end of the assigned incubation period, washed with distilled water, and dried with absorbent paper. Surface microhardness test was performed using a microhardness tester with a Knoop indenter (Tukon 300, Acco Industries Inc., Wilson instruments division, Bridgeport CT, USA). Three Knoop Hardness Number (KHN) readings were recorded for each specimen under 100 g loads for 30 seconds. This load created a clear and reliable indent in all materials.

**Compressive strength:** Specimens (6 mm diameter and a length of 14 mm) were used (n=5/material for each group), the materials were placed in the molds and transferred to a 95-100% humidity chamber and incubated at 37°C for 72 h. Surfaces of the specimens were wet polished with fine sand paper (600-1200-grit; 3M, St Paul, MN, USA) to achieve a flat surface that is perpendicular to the long axis of the cylinder with a uniform specimen length of 12 mm [68]. Samples from each dental material were divided randomly into three subgroups: A) no incubation, B) 28 days in PBS, or C) in SHSE, at 37 °C. The cylinders were tested in compression using a universal testing machine (Instron Corp, Canton, MA, USA) at a constant crosshead speed of 0.5 mm/min. The maximum load necessary to crush the specimen was recorded.

To calculate the compressive strength (C) the following formula was used:

\[ C = 4P/\pi D^2 \]

P is the failure load and D the diameter of the tested specimen.

**Dimensional change (DC):** Cylindrical test specimens (n=5/material in each group), with a diameter of 6 mm and a height of 12 mm were made [358]. Five minutes after the start of mixing, the molds with clamps were transferred to a cabinet with 95–100% relative humidity and kept at 37°C. After 72 h, the ends of the test specimens were ground flat on wet silicon carbide paper (600-1200-grit; 3M, St Paul, MN, USA) before being removed from the mold. The initial length of each specimen was measured with a digital caliper (Mitutoyo Digital Caliper, Japan) and recorded as L1. Specimens were
then stored in either PBS or SHSE at 37 °C for 7 or 28 days. After that, the specimens were lightly dried using absorbent paper, and the final length was determined (L2). The percentage of dimensional change (DC %) was calculated as follows: 

$$DC = \frac{(L_1 - L_2)}{L_1} \times 100$$  [362, 363].

**Solubility test/ (Weight loss (WL)):** Specimens (20 ± 0.1 mm diameter, height 1.5 ± 0.1 mm) were prepared. All specimens were left to set for 72 h by incubation in 100% relative humidity at 37°C. After this period, the specimens were finished to remove flash and irregularities, then inserted in a dehumidifier chamber for 1 h to remove any moisture from the surface, each sample was weighed three times (Shimadzu Corporation, Japan) and the average weight reading was recorded as W1. Afterward, the samples in each group were randomly divided into four subgroups (n=5/group): A) 7 days in PBS, or B) SHSE, C) 28 days in PBS, or D) PBS. Samples were removed at the end of the assigned incubation period, washed with 2 ml of distilled water, dried with absorbent paper, and placed in a desiccator for 24 h. The samples were weighed three times and the average weight reading was recorded as W2. The percentage of solubility was calculated according to the following formula: 

$$\text{Solubility (WL%)} = \frac{(W_1 - W_2)}{W_1} \times 100$$  [364].

**Statistical Analysis:**

After testing the normal distribution, data were analyzed by three-way ANOVA followed by Tukey’s test for repeated-measurement pair-wise comparison within the material type, incubation medium, and time for their effect on the physical properties (microhardness, compressive strength, weight loss, and dimensional change) (p<0.05). All analysis was performed using the Statistical Package of Social Science (SPSS Inc., Chicago, IL, USA).

**Results:**

**Microhardness**

Following incubation in either PBS or SHSE, the microhardness of BC increased, and of RC decreased with time compared to the baseline measurements. Neither PBS, nor SHSE incubation had a significant effect on the microhardness of ER throughout the incubation period (Fig. 4.1). RC exhibited the highest microhardness of all tested materials (p<0.001) for all tested conditions. Microhardness of BC after 7 days in PBS (38.1±7.9
KHN), and 28 days in SHSE (40.8±7.4 KHN), and PBS (43.9±6.7 KHN) was significantly (p<0.001) higher compared to the baseline (24.9±6.3 KHN). However, SHSE reduced BC microhardness (28±4.8 KHN) compared to PBS (38.1±7.9 KHN) after 7 days of incubation (p>0.005). SHSE incubation reduced the microhardness of RC (55.6±7.1 KHN) compared to PBS (66.3±6.5 KHN) and to the baseline (71.7±4.7 KHN) after 7 days of incubation (P<0.005, 0.001 respectively). After 28 days SHSE incubated groups (52.3±9.2 KHN) was significantly (p<0.05) less than PBS incubated groups (62.6±8.5 KHN) and both were significantly less than the baseline (p<0.001, 0.05 respectively).

**Compressive strength:**
The results of the compressive strength measurements are depicted in (Fig. 4.2). RC had the highest compressive strength among the three materials (p<0.001) with average range from 207±20.4 to 235.4±17.6 MPa, followed by ER (51.5±1 to 59.1±7.5 MPa), and the least was for BC (13.1±1.5 to 19.1±2.1-MPa). BC had significantly increased compressive strength values over the 28 days incubation period compared to the baseline in both incubation media (p<0.005 for PBS, and p<0.001 for SHSE), but it remained the lowest (p<0.001). The 28-day incubation in SHSE and PBS had no significant effect on the compressive strength of ER, and RC.

**Dimensional change:**
The Dimensional change DC% results are shown in (Fig 4.3). BC sealer expanded with mean ranging from (0.03-0.08%) over the 28 days, the expansion was significantly (p<0.001) less after 7 days for SHSE (0.03±0.01%) incubated samples when compared to PBS (0.05±0.01%) at the same time period. RC exhibited shrinkage (0.38-0.57%) that increased significantly (p<0.05) after 7 days of incubation in SHSE compared to PBS (0.5±0.07% vs. 0.38±0.08%). For ER, the shrinkage was very low ranging from 0.05±0.01 to 0.06±0.01% for all groups and was not affected by the incubation media.
Solubility/weight loss:
The weight loss of BC sealer was the highest with mean ranging (1.75±0.34%-2.96±0.189%), followed by RC (0.54±0.07%-0.89±0.03%), while ER showed the least weight loss (0.11±0.1%-0.12±0.12%) (Fig. 4.4). Incubation in SHSE increased the solubility significantly (p<0.05) compared to PBS for BC after 28 days of incubation (2.96±0.189 vs. 2.4±0.2, respectively) and after 7 days for RC (0.8±0.1 vs. 0.54±0.1, respectively).
Discussion
In the absence of proper coronal seal, saliva can cross the root canal leading to root canal treatment failure [348]. Therefore, it is imperative to investigate any detrimental effect of salivary enzymes on the root canal sealers’ physical properties. The present study assessed selected root canal sealers physical properties that are related to their clinical performance when in contact with PBS or SHSE using experimental techniques that have been recommended for standard testing of endodontic materials [356-358]. SHSE have shown to accelerate the degradation of dental methacrylate resins [365], but its effect on the root canal sealers has not yet been investigated. The samples were exposed to the enzyme for up to 28 days as most of the physical changes are expected to occur during the first 28 days after setting [23-25, 343].

ER has been used as a gold standard in comparative studies of root canal sealers due to its favorable physical, biological and antimicrobial properties [35, 344]. BC sealer is an example of a calcium phosphate silicate–based cement, described as insoluble, premixed hydrophilic cement, that can tolerate moisture and small amounts of blood, and have good antimicrobial, sealing, and mechanical properties [345]. The RC used in this study is a methacrylate resin self-cured polymer that has the potential to be used as a sealer for root canal filling [346, 347], as well as a coronal restoration [187, 188], due to its ability to bond to the root canal dentin and improve sealing capacity of the filling. In the current study ER showed that it is the most stable to moisture and salivary esterases, and this could be explained by being hydrophobic and does not contain ester bonds, its stability in solutions confirm what was found previously in other studies [366]. When compared to the other two materials it had the lowest solubility and dimensional change, microhardness almost comparable to BC and less than that of RC, and a compressive strength in between the two materials.

Surface microhardness is an indicator of the setting process of the material [367], and a significant parameter of root canal sealer to resist applied occlusal stresses within the canal [368, 369]. Hardness can be used as an indicator of the hydration process of hydraulic cements [370], and the polymerization extension of dental resins [41, 371,
In this study, the surface microhardness of BC, ER, and RC were evaluated after incubation in PBS or SHSE for 7 and 28 days. The results demonstrated that SHSE exposure had a detrimental effect on the surface microhardness of BC and RC. The hydration process and the crystalline structure of BC are affected by the environmental conditions and this could have an adverse effect on the surface hardness of the cement [350, 373]; the proteins in SHSE might be adsorbed to the cement reducing the water uptake, thus delaying the setting and reducing the hardness of BC [31, 374]. Methacrylate resins are vulnerable to hydrolysis due to the presence of ester linkages within its monomers [44, 52, 335]; the hydrolysis process can be further catalyzed by the presence of esterase enzymatic activity in saliva and bacteria [52]. RC showed reduced microhardness, that could be explained by water sorption and hydrolytic reaction that is catalyzed by esterases in SHSE [52]. It is expected that in the oral cavity, where chewing forces and the presence of food, this process will be even more accelerated for this type of materials [375].

In addition to the testing of surface microhardness, the compressive strength of the materials was examined. Higher compressive strength values make root canal sealers more durable and resistant to the displacement of cones during post and core placement [376]. Clinically the accelerated setting time is a desirable property of endodontic materials [375], compressive strength is used to monitor the setting process and it is of greater value, as some material strength continues to increase when the setting time is recorded [377]. According to the results of this study, the compressive strength of the three materials was not affected by the incubation in SHSE. BC had increased strength with time and this was reported by other studies [378]. This could be explained by continuing hydration and crystallization of the calcium silicate-based cements for up to 4 weeks [35, 378]. Microhardness values for BC were lower than what was reported for other studies [35], and this could be explained by the need to immerse one side of the samples in water during setting in order to get hard samples, and this process was hard to standardize the amount of the absorbed water under in vitro conditions and could have adversely affected the compressive strength [379]. RC compressive strength was comparable to the values that were reported previously [365]. No minimum compressive
strength requirement for root canal sealers is reported in the literature, therefore the higher compressive strength of RC does not give a conclusion of a better performance when compared to ER, and BC.

Excessive shrinkage and expansion are not desirable properties for a root canal filling material. Shrinkage of non-bonded sealers produces pathways for bacteria and their product [380], and could cause internal stresses, while expansion may elicit cracks in the root [363]. The dimensional change of BC, RC and ER was in agreement with ISO 6876/2012 specifications, which recommends that dimensional change for root canal sealer should not exceed 1.0% shrinkage or 0.1% expansion. BC showed slight expansion, the expansion was less for SHSE incubated group at 7 days compared with PBS incubation, that could be explained by reduced water uptake due to the protein filling the porosities within the cement [381]. This slight expansion may contribute to superior sealing ability. Thus, further tests are required to ascertain if BC effectively seals root canals without increasing the risk of development of cracks or root fracture. RC and ER showed shrinkage over the 28 days regardless the incubation media; the shrinkage was higher for RC after 7 days in SHSE vs. PBS. RC and ER contain polymers, therefore polymerization shrinkage is expected upon material setting [382]. RC is a combination of inorganic filler particles coated with coupling agents, and are dispersed in an organic methacrylate resinous matrix. Before the polymerization process, van der Walls forces act and keep the monomers grouped. During the polymerization process, these forces are substituted by covalent bonds; consequently, volumetric shrinkage occurs [383].

High solubility of root canal sealers is undesirable and has been linked to the irritation of periapical tissues [187], and may increase the sealer-dentin interfacial bacterial leakage over time [322, 323]. All tested materials showed solubility within the limit allowed in the ISO 6876/2012 recommendations (3% mass fraction). Monomers and degradation by-products from the materials could affect gene expression and protein of the bacteria [210]. A more porous matrix may be produced when the sealer sets in the presence of moisture in wet canals, which, in turn, may result in increased leaching of tissue-irritating substances from the set sealer [368]. SHSE increased the solubility of BC at 28 days and
this could be explained by the improper crystallization of the cement that makes it more vulnerable to hydrolysis and/or dissolution by the media [52]. The increase of RC solubility following 7 days of incubation in SHSE could be a result of ester links hydrolysis and release of byproducts [31, 33, 43, 52, 53, 335, 384, 385]. Many studies have shown the ability of esterases to degrade dental composites indicated by the release of the biodegradation product Bis-hydroxypropoxy-phenyl propane (BisHPPP), adversely affecting the bond between composite and tooth structure, and increasing the depth of bacterial proliferation between the tooth-composite interface [33]. The process is slower in the absence of esterases, but after 28 days, both PBS and SHSE contribute similarly to the weight loss of RC. The number of readily accessible ester linkages within the resin matrix gradually declines over time [334] modulating the effect of SHSE on weight loss.

**Conclusions:** Within the limitations of this laboratory study, the results showed variability of the physical properties between the three sealer materials. ER physical properties were not affected by moisture and enzyme exposure. The material showed the least weight loss and dimensional change, and acceptable microhardness and compressive strength. RC had the highest microhardness and compressive strength, but it had the greatest shrinkage, and some of its properties were adversely affected by the presence of SHSE. BC was the only material that slightly expanded upon aging, however the higher dissolution rate and susceptibility to the presence of SHSE could impact its clinical performance. In general, materials used in the current study showed solubility and dimensional changes within the limitations of ISO (6876/2012). This highlights the importance of using incubation media with enzymatic activities like those that exist in human saliva rather than the common use of buffers or water as incubation media for the assessment of aging on the material properties. As some physical properties continued to change over the 28 days incubation period, longer periods of incubation might be needed to have a better idea of the materials’ performance in both PBS and SHSE.
Figure 4.1: Schematic diagram showing changes in microhardness values (KHN) for the test groups (BC, ER, and RC) after being exposed either to PBS or SHSE for different time periods. Data are shown as mean ± SD. Different letters represent statistical significance between different materials at different incubation periods and media.
Figure 4.2: Compressive strength values (in MPa) for BC, ER, and RC at zero-time, 28 days incubation in PBS, or SHSE. Data are shown as mean ± SD. Different letters represent statistical significance between the different materials at different incubation times and media.

Figure 4.3: Dimensional change as percentage of baseline for (BC, ER, and RC) after being exposed either to PBS or SHSE for different time periods. Data are shown as mean ± SD. Different letters represent statistical significance within the same material at different incubation periods and media.
Figure 4.4: Mean weight loss and standard deviations of BC, ER, and RC after 7, and 28 days incubation in either PBS or SHSE. Data are shown as mean ± SD. Different letters indicate significant difference between materials at different incubation times and media (p<0.05).
Chapter 5

Enterococcus faecalis Hydrolyzes Dental Resin Composites and Adhesives

Muna Q. Marashdeh1, 2, Russel Gitalis1, 2, Celine Lévesque1, Yoav Finer1, 2

1Faculty of Dentistry, 2Institute of Biomaterials and Biomedical Engineering
University of Toronto

Abstract

Background: Post root canal treatment, the dentin-sealer interface undergoes degradation, allowing for interfacial microbial biofilm proliferation and treatment failure. Saliva and cariogenic bacteria showed esterase-like activities; cholesterol esterase-like (CE) and/or pseudocholin esterase-like (PCE) that degrade methacrylate-based resin composites and the restoration-tooth interface, increasing microbial biofilm interfacial ingress and proliferation. Enterococcus faecalis is a Gram-positive bacterium that is commonly detected in persistent endodontic infections. The aim of this study was to measure E. faecalis esterase-like, CE-like and PCE-like activities, and to assess the ability of the bacterium to degrade methacrylate-based resin composite, total-etch, and self-etch adhesives. Methods: CE-like and PCE-like activities from E. faecalis were measured using nitrophenyl and butyrylthiocholine substrates, respectively. The ability of E. faecalis to degrade resin composite (RC), total-etch (TE), and self-etch (SE) adhesives was examined by quantifying the release of bis-hydroxy-propoxy-phenyl-propane (BisHPPP), a universal resin degradation byproduct, using high performance liquid chromatography. Results: E. faecalis expressed CE-like (1.23±0.13 Units/μg dry bacteria), but no PCE-like activity. After 30 days and/or 14 days of incubation the amount of BisHPPP released was significantly higher in the presence of bacteria vs. media for TE adhesive and RC but not SE adhesive (p<0.05). The amount of BisHPPP released after 30 days of incubation with bacteria was significantly different between RC (3.43±1.20 μg/cm²), TE (23.69±1.72 μg/cm²) and SE (0.86±0.44 μg/cm²) adhesives (p<0.05). Conclusions: E. faecalis possesses esterase-like degradative activity towards dental methacrylate resin restoration materials, which could accelerate the degradation of
the dentin-methacrylate resin interface, increasing bacterial biofilm proliferation and penetration into the root-canal system.

**Key words:** *Enterococcus faecalis*, esterases, biodegradation, methacrylate resin.
Introduction

Methacrylate resin based endodontic sealers were developed since they could bond to the root canal walls [25]. However, no added advantages were noticed over the use of the conventional sealers and it was difficult to establish a reliable bond between the root canal dentin and the resin material [58, 59]. The use of methacrylate resins nowadays in root canal is mainly limited to post cementation [60] and coronal restorations of endodontically treated teeth [386], thus their ability to maintain interfacial seal is still important to the success of the endodontic treatment. Resin composite (RC) materials require the application of resin adhesive systems in order to bond to dentin. Two main adhesive systems are currently available: etch and rinse (total etch (TE) technique that is designed to remove the smear layer and self-etch (SE) technique that modifies the smear layer [387]. Both are dependent on the formation of a hybrid layer [31, 385]. Methacrylate resin polymers in restorative material and adhesives are prone to hydrolysis due to the presence of unprotected ester linkages [52]; salivary [44] and bacterial esterases [388] can catalyze this process, increasing the degradation of the resin-dentin interface at a greater rate and increase the bacterial proliferation into the interface [64]. Bacterial infection caused by coronal leakage is an important cause of endodontic failure [389].

Bacterial enzymes are considered an important part of bacterial pathogenicity [390], and could cause tissue destruction [391]. Bacterial esterases have been linked to the virulence of Mycobacterium tuberculosis as they hydrolyzed the free esters in the local environment and accordingly could be a source of fatty acids to be used nutritionally by the bacteria [392]. Esterase from Group A Streptococcus has been found to contribute to severe invasive infections [44]. More recently, esterase activity from the cariogenic bacteria Streptococcus mutans has been found to hydrolyze dental methacrylate resins and therefore these bacteria have the potential to degrade the restoration-tooth interface, and further increase bacterial biofilm proliferation and restoration failure [65-67]. E. faecalis is a Gram-positive, facultative anaerobic coccus that has been frequently isolated from canals with persistent periapical infections [61]. It enters the root-filled canals via coronal leakage during or after root-canal treatment [68], this ability of
E. faecalis to invade root canals and cause infections has been linked to many virulence factors [141], such as its ability to adhere to dentin [68, 123], and to survive and grow in harsh environments [44, 52]. The hypothesis of this study is that E. faecalis has esterase-like activity in levels that enables the bacteria to degrade methacrylate resin composites and adhesives.

Methods

Bacterial esterase activity assays: Overnight (o/n) culture of E. faecalis ATCC 29212 was grown in brain heart infusion (BHI). Part of the o/n culture was heat inactivated (HIN), for 30 min at 80°C. The total effect of HIN treatment on bacterial viability was verified by serial dilution and determination of CFU. The other part was diluted 1:20 and incubated (37°C) until reaching different growth phases (lag, log, and stationary), then bacterial suspension for each growth phase and HIN were centrifuged (13,000 RPM, 10 m), the bacterial culture supernatant was collected and filtered through a 0.22-μm-pore-size filter to remove residual bacterial cells and the bacterial cells were resuspended in phosphate-buffered saline (PBS) (pH=7.0). One mL of the bacterial suspension and the culture supernatant (to detect enzymes released into the environment) were incubated with either 0.5 mL of p-nitrophenylbutyrate (p-NPB), or p-nitrophenylacetate (p-NPA) to measure the acetate-like dependent esterases, or butyrylthiocholine iodide (BTC) substrates to measure the butyrate-like dependent esterases (Sigma, St. Louis, MO) and the esterase, CE-like and PCE-like activities were quantified with a spectrophotometer at 401, 405 nm, respectively as described previously [44]. Absorbance values were normalized to bacterial suspensions without substrates absorbance readings.

Biodegradation of composite and adhesive: Photopolymerized samples (2x4x4 mm) (N=3/each material, incubation condition and time period) were made from RC (Filtek™ Z250), TE adhesive (Scotchbond™ Multipurpose), and SE adhesive (Adper™ Easybond) (all materials from 3M Canada Inc., London, ON) as previously described [44] and incubated in sterile vials containing 2 mL BHI (control), or 1:20 diluted E. faecalis ATCC 29212 o/n in BHI (experimental) for 2, 4, 8, 14, and 30 days. Incubation solutions were collected and replaced every 48 h. When collected, solutions
were mixed with equal amounts of methanol (100%) to halt enzymatic activity, then filtered (Amicon Ultra centrifugal filters, 14,000 RPM for 10 m at 4°C) and refrigerated at 4°C in a well-sealed Eppendorf tubes [33]. High performance liquid chromatography (HPLC) was used to quantify bis-hydroxy-propoxy-phenyl-propane (BisHPPP) [43, 44, 387], a bisphenol-glycidyl-dimethacrylate (BisGMA) universal degradation byproduct [43, 44, 387]. The amount of BisHPPP released was normalized to the surface area of the specimens (mm²) [43, 44, 387].

**Surface morphology:** Observation for pre- and post-incubation specimens were performed using scanning electron microscopy (SEM) (Hitachi S 2500 SEM, Mito City, Japan) at an operating voltage of 10 kV [43, 44, 387]. Specimens were sonicated prior to analysis to remove bacterial cells adhering to the surface, followed by sample dehydration and gold coating then imaging [52].

**Statistical analysis:** After testing for normal distribution, data were analyzed by three-way ANOVA followed by Tukey’s test for repeated-measurement pair-wise comparison within the growth phase, type of substrate, bacteria or supernatant for their effect on the esterase activity level, and a three-way ANOVA followed by Tukey’s test for repeated-measurement pair-wise comparison within the incubation media, time and material for their effect on the amount of BisHPPP release (p < 0.05).

**Results**

**Bacterial esterase activity assays:** All growth phases of *E. faecalis* had activity towards both nitrophenyl esters (p-NPA, and p-NPB) but not toward BTC. At each growth phase, the preference towards the p-NPA substrate was higher than p-NPB with the highest activity observed in the log growth phase (p<0.05), at 1.23±0.13 Units/μg cell dry weight. For all substrates, the HIN and culture supernatant showed significantly lower activity for all growth phases compared to the bacteria (live samples). *E. faecalis*, HIN, and the culture supernatant did not show activity towards the BTC substrate at all growth phases (Fig. 5.1).
**Biodegradation of composite and adhesives:** For all materials (RC, TE, and SE), a trend of increasing BisHPPP released throughout the incubation period was observed (Fig. 5.2). After 30 days of incubation with resin composite, and 14 and 30 days incubation with TE but not SE, the amount of BisHPPP released was significantly higher in the presence of bacteria compared to control (p<0.05). After 30 days of incubation with *E. faecalis* the amount of BisHPPP released from TE (23.69±1.72 μg/cm²) was 7 and 28 times higher than that released from RC (3.43±1.20 μg/cm²) and SE (0.86±0.44 μg/cm²), respectively (p<0.05). The total amount of BisHPPP released in the samples incubated with bacteria over the entire incubation period was significantly higher in TE (97.78±9.40 μg/cm²) compared to RC (17.28±1.53 μg/cm²) and SE (5.31±0.42 μg/cm²) (p<0.05).

SEM micrographs (Fig. 5.3) demonstrated that the surface of the specimens incubated with *E. faecalis* for 30 days appear rougher than BHI-incubated and non-incubated specimens.

**Discussion**

The results of this study support the hypothesis that *E. faecalis* ATCC29212 contains esterase-like activity at levels capable of degrading cured methacrylate resin composite and adhesives. This finding shows a potential ability of *E. faecalis* strains with esterase activity to invade and penetrate the interface between methacrylate resin materials and tooth structure to enter the root canal, and cause secondary infections. The bacterial enzymes showed higher activity toward degrading total-etch adhesive, followed by resin composite, and the least activity was toward self-etch adhesive.

Human saliva [44] and *S. mutans* species [52] have been shown to hydrolyze resin composites and adhesives. Human salivary esterases have been characterized by their activity toward nitrophenyl esters and BTC [44], while *S. mutans* had characteristic activity towards the nitrophenyl esters, but not toward BTC. Strains of *S. mutans* showed different affinity toward the nitrophenyl substrates [43, 275]. In the current study *E. faecalis* expressed minimal activity toward BTC and a higher activity toward nitrophenyl esters, and this was in levels similar to several strains of *S. mutans* that were
shown previously to degrade resin composites and adhesives [31, 42-44, 53]. *E. faecalis* activity levels and profile were closer to that of *S. mutans* than that of salivary esterases, specifically JH1005, LT11, UA140, and BM71 strains that are linked to dental caries with preference toward p-NPA more than p-NPB.

Resin composite (RC, Z250, 3M), total etch adhesive (TE, Scotchbond™ Multipurpose, 3M), and self-etch adhesive (SE, Adper™ Easybond, 3M) were selected in this study as they are commonly used materials in many dental practices, and since these materials were extensively investigated, there are good baseline data regarding their biostability [387]. These materials are good representatives of RC, TE, and SE group of materials so the results of this study could be generalized to similar materials from each group. All three materials are BisGMA-based which makes the analysis of the degradation and comparison feasible and standardized [33, 43, 44, 385, 387].

BisHPPP is a biodegradation by-product of BisGMA, a universal monomer present in most adhesives and composite materials. BisHPPP was chosen for analyses since it is a good marker of true resin biodegradation due to the hydrophobic nature of its precursor [44]. *E. faecalis* significantly increased the amount of BisHPPP released for RC and TE adhesive after 30 and/or 14 days of incubation, while there was significantly lesser effect on the SE material compared to the control. The total amount of BisHPPP released in the samples incubated with *E. faecalis* over 30 days was significantly higher in TE adhesive compared to RC and SE adhesive. The percent composition of BisGMA of total-etch adhesive, self-etch adhesive and composite are 60-70, 15-25, and 5-10% of the total mass of the materials, respectively [3]. Since the amount of BisHPPP released was not correlated with the BisGMA content per material, and since all materials were similar in their surface area, volume, and degree of vinyl group conversion [393], this difference could have arisen only from the inherent variations in chemical composition of the materials and their interaction with the specific enzymatic activities of *E. faecalis*.

Susceptibility of the polymers to microbial enzymatic attack generally depends on enzyme availability, enzyme specificity for a polymer, availability of a site in that
polymer for enzyme attack, and the presence of coenzyme if required [44]. Different biodegradation profiles were found for *S. mutans* UA159 vs. *E. faecalis* ATCC 29212. *S. mutans* UA159 showed different preferences than *E. faecalis* ATCC 29212 toward the nitrophenyl substrates, with preferences toward pNPB compared to pNPA as well as differences in the degradation of the restorative materials, RC, TE and SE adhesives [394, 395]. This further suggests that specific enzymatic activities from each species are responsible for the observed activity profiles and degradative activities. Variations in the enzymatic activities are expected due to the difference in the esterase genes between different species [44, 396-398].

Following 30-days of incubation, SEM analysis showed increased surface roughness for all incubated samples compared to the non-incubated samples, indicating the degradation of all materials. This observation corroborates previous studies that showed surface degradation and changes in surface-topography of polymer-based restorative materials by bacteria [44]. However, except for Bourbia et al. [399], these studies provided only observation of surface changes without characterization of possible degradative activities from the bacteria. The current study is the first to characterize esterase activities by *E. faecalis* that degrade methacrylate-based restorative materials.

Esterase-like activities were different for each growth phase of the bacteria, with the lag phase having the lowest enzymatic activity followed by the stationary phase and the highest values were for the log phase. This could be explained by the physiology of each phase; the lag phase allows the adaptation required for bacterial cells to begin to exploit new environmental conditions [400], and the cells are metabolically less active than the other phases [399]. In the log phase cell division proceeds at a constant rate and it is considered as the most active metabolically [401], while the stationary phase starts when conditions become unfavorable for growth and bacteria stop replicating and reduced its metabolism. The transition between the log and stationary phases involves a period of unbalanced growth during which the various cellular components are synthesized at unequal rates. Consequently, the difference in the chemical composition from those phases could have led to the difference in the activity noticed in the current study [402].
The heat inactivation (HIN) process affects the structural and physiological properties of every cellular component of the bacteria (membrane, enzymes and proteins, DNA, and RNA) [403, 404]. Therefore, this process is useful to establish a group of inactive bacterial suspensions to act as negative control to which the live bacterial suspensions can be compared [405]. In the current study HIN E. faecalis showed a significant decrease in the esterase-like activity of the bacteria. Since the pH and the growth conditions were similar for the two bacterial cultures, it could be hypothesized that the measured activity is due to true enzymatic activity from the bacteria and not due to other factors, such as low pH, or the incubation media.

The supernatant had less esterase activity than the bacterial suspensions under the conditions of the current study. However, the lower enzymatic activity of the supernatant does not exclude the possibility of the enzyme being secreted, as the bacteria utilize several methods to invade mammalian hosts, damage tissue sites, and thwart the immune system from responding [406], including transport of proteins from the cytoplasm across the inner membrane into the bacterial supernatant or onto the surface of the bacterial cell [407]. Furthermore, bacterial species in nature have a strong tendency to form complex biofilms, bacterial cell death and lysis are important for biofilm development, and have an essential role in intercellular adhesion and biofilm stability [408]. The release of cell contents, nutrients and enzymes, such as those involved in the process of hydrolysis of dental materials, and hence contributing to the continued survival of the biofilm population as a whole [33]. Overall, the fact that the bacteria hydrolyzed the resinous materials in the current study confirms that enzymes were either released or attached to the cell wall, though the exact mechanism and identity of the enzyme require further investigation.

Kermanshahi et al. [15] showed that exposure of dentin-composite restorations to salivary esterase-like activity resulted in the formation of gaps that were infiltrated and colonized by bacterial biofilms. E. faecalis could contribute to the deterioration of the resin-dentin interface when present within the restoration-tooth marginal gap by releasing both protease collagenase enzyme (chapter 6) and esterases, affecting the hybrid layer, tooth
and composite, and potentially compromising the integrity of the margins, reducing the longevity of the restoration, increasing the bacterial proliferation into the root canal system, and contributing to the failure of root canal treatment [31, 241, 409]. Increasing the esterase resistance of adhesives and of methacrylate composites could potentially improve the materials biochemical stability, and extend the longevity of resin-dentin bonds, therefore increasing the lifespan of the dental resin restorations [43, 44, 52].

**Conclusions:** Overall, the activity patterns of *E. faecalis* suggest that it could be a contributor to acetate-like dependent esterase activities of saliva and less to the butyrate-like dependent esterases that are characteristic of human salivary esterase activity [62]. The extent of dental methacrylate resin restorations and adhesives degradation by *E. faecalis* was material dependent, and material chemistry was the most important factor in determining the material’s biochemical stability. When present within the confined space of the restoration-tooth marginal interface *E. faecalis* could contribute to the deterioration of the resin-dentin interface accelerating its proliferation into the root canal potentially compromising the success of root canal treatment.
Figures

**Figure 5.1**: Activity profile of *E. faecalis*, *E. faecalis* culture supernatant at lag, log, stationary phase, and of HIN with p-nitrophenylacetate (p-NPA), p-nitrophenylbutyrate (p-NPB). Data are shown as mean ± SD (N=3). Different letters indicate significant difference between activities (p<0.05).
**Figure 5.2:** Biodegradation study: cumulative BisHPPP release from resin A) composite, B) total-etch, and C) self-etch adhesives. Data shown as mean ± SD. *E. faecalis* increased the amount of degradation indicated by increased BisHPPP compared to medium (BHI) only for resin composite after 30 days of exposure and for total-etch adhesive after 14, and 30 days of exposure. Different letters indicates significant difference between the same incubation groups at different time points, and media (*P*<0.05). Composite and SE have same Y-axes scale, which is different from TE.
Figure 5.3: SEM micrographs (x10K original magnification) of representative images of the surface of resin composite, self-etch, and total-etch adhesives samples before incubation and 30 days after incubation in either BHI, or *E. faecalis*, show degradation of material.
Enterococcus faecalis possesses Collagenolytic Activity that Degrades Human Dentin Collagen Matrix

Muna Q. Marashdeh¹,², Russel Gitalis¹,², Celine Lévesque¹, Yoav Finer¹,²
¹Faculty of Dentistry, ²Institute of Biomaterials and Biomedical Engineering
University of Toronto

Abstract

Background: Enzymatic activities from interfacial bacteria could contribute to the degradation of dentinal collagen fibrils, undermining the sealer-dentin interface integrity, and causing an increase in interfacial biofilm proliferation, which is a major reason for post-endodontic failure. Enterococcus faecalis is a bacterium that is commonly isolated from persistent endodontic infections. The aim of this study was to measure E. faecalis collagenolytic protease activity and its ability to degrade human dentinal collagen matrix.

Methods: Proteases activities of E. faecalis ATCC 29212 toward generic and specific human matrix metalloproteinases (MMPs) substrates were measured using fluorimetric assay. The ability of E. faecalis to degrade dentinal collagen was tested by quantifying the amount of hydroxyproline released to the media following incubation of the bacteria and the heat inactivated bacteria (HIN) with demineralized human samples for 24 h and by scanning electron microscopy (SEM).

Results: E. faecalis showed generic and specific MMP -1, -2, -8, and -9 like activities with mean±SD of 5.17±0.47, 4.07±0.14, 1.32±0.24, 7.75±0.88, and 5.98±0.61 μM/3*10⁶ CFU, respectively. The amount of hydroxyproline released from demineralized dentin by E. faecalis (1.8±0.17 μg/50μl) was significantly higher (p<0.001) compared to HIN (0.61±0.22 μg/50μl). SEM showed increased collagen network degradation after incubation with E. faecalis vs. HIN.

Conclusions: E. faecalis possesses collagenolytic protease activity that enables the bacteria to degrade dentinal collagen, potentially compromising the interface and tooth structure. The presence of collagenolytic protease activity may facilitate the migration of
*E. faecalis* from the root canal into the periapical lesion and explain part of Enterococci role in apical periodontitis.

**Key word:** *Enterococcus faecalis*, collagenase, collagen degradation, human dentinal collagen, matrix metalloproteinases (MMP), hydroxyproline.
Introduction
Collagen is the major protein found in dentin, it constitutes ~90% of the organic matrix, most of which is type I collagen [62]. Dentinal collagen serves a variety of structural roles including shaping and organizing extracellular matrices [287], providing a scaffold for minerals formation and deposition [288], cells adhesion and differentiation [289], and preserving the structural, mechanical and functional integrity of the dentin [51].

Bacterial infection caused by coronal leakage is a significant contributor to endodontic failure [64]. Bacterial pathogenicity includes secretion of several enzymes that causes tissue and material destruction that is dependent on type of enzyme, availability of a site in the substrate for enzyme attack, and enzyme specificity for different substrates [389, 393]. Bacterial collagenases include metalloproteinases and serine proteases that are involved in the degradation of the extracellular matrices of animal cells, due to their ability to digest native collagen. Collagenase enzymes are important virulence factors in a variety of pathogenic bacteria [50, 299], and as such may contribute to the degradation of collagen fibrils and deterioration of structural integrity in root dentin with time [63].

*Enterococcus faecalis* is a Gram-positive, facultative anaerobic coccus that has been frequently detected from patients with persistent periapical infections [65-67]. It enters the root-filled canal via coronal leakage during or after root-canal treatment as secondary invaders [61]. The ability of *E. faecalis* to cause infections has been linked to numerous virulence factors [68]; it is able to adhere to dentin [410], survive, and grow in harsh environments [68, 123]. The aim of this study was to measure *E. faecalis* collagenolytic protease activity and its ability to degrade human dentinal collagen matrix. The hypothesis was that *E. faecalis* possesses a collagenolytic activity that enables it to degrade the dentinal collagen.

Methodology
**Generic and specific collagenolytic, MMP-like activities from *E. faecalis***: MMP-like activity was determined using generic and specific MMP-1, -2, -8, and -9 Assay Kits (SensoLyte® 520 Generic MMP Activity Kit *Fluorimetric*, SensoLyte® 520 MMP – 1,
2, 8, and Assay Kit *Fluorimetric*, AnaSpec, San Jose, CA, USA) following the manufacturer’s instructions [411]. These kits detect MMP activity using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMP, the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission wavelengths of 490/520 nm. The procedure included preparation of generic and specific -1, -2, 8, and -9 MMP substrates (5-FAM/QXL™520), fluorescence reference standard (5-FAM-Pro-Leu-OH), and one of MMP (-1, -2, -8, or -9) as a positive control. Overnight (o/n) cultures of *E. faecalis* ATCC 29212 and heat inactivated *E. faecalis* (HIN) were prepared using chemically defined media (CDM). HIN culture was prepared by heating bacterial suspension for 30 min at 75 °C in a water bath [412]. The total effect of HIN treatment on bacterial viability was verified by serial dilution and determination of CFU. Bacterial suspensions were centrifuged (13,000 RPM, 10 min), re-suspended in the assay buffer provided with the kit, and incubated with the generic and specific -1, -2, -8, and -9 substrates for 60 min to give sufficient time for the enzyme to cleave the substrate. The assays were performed in a 96-well plate, and the activities were quantified using fluorimetric plate reader (Cytation Multi-Mode Reader, BioTek, Vermont, USA). Fluorescence values were normalized to assay buffer with the substrate (background). All experiments were performed in triplicate.

**Human dentinal collagen matrix degradation:** Intact freshly extracted human molars kept at −20 °C were used (University of Toronto ethical approval protocol #2014-28214). Crowns were cut using a slow speed diamond saw (Isomet, Buehler, Lake Bluff, IL) under water lubrication to obtain dentin slabs of (6x6x1 mm, WxLxT). Dentin specimens were demineralized in 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 18 h to expose the organic matrix [413], then, neutralized with 10% sodium ascorbate, washed with distilled H₂O, dried, and sterilized with 70% ethanol for 15 min. Dentin specimens were then transferred to either 1:20 o/n of *E. faecalis*, HIN *E. faecalis* (control), or Collagenase Type I (*Clostridium histolyticum*, Gibco®, Sigma-Aldrich) at 125 U/mL (positive control). CDM only used as a negative control. After 24 h of
incubation, dentin specimens were removed and processed following a method described previously by Carvalho et al. [414] and analyzed using scanning electron microscope (SEM) (JEOL, USA Scanning Electron Microscopy, model 6610LV). The supernatant of each sample was filter centrifuged for 10 min to remove any remnants of bacterial cells and large proteins that might affect the hydroxyproline analysis, and was analyzed for hydroxyproline content at the Advanced Protein Technology Center of The Toronto Hospital for Sick Children using the method described by Bidlingmeyer et al. [415]. Free amino acid analysis was performed on a UPLC system (Waters Acquity, Waters Corporation, Milford, MA). Sample aliquots were transferred into glass culture tubes and dried under vacuum. The samples were derivatized for 20 min at room temperature with a derivatizing solution. The derivatized amino acids were detected at 254 nm. Data were collected, and processed by using Waters Empower 2 Chromatography software [416]. All results were normalized to values from samples incubated in CDM only.

Statistical methods and outcome measures

After testing for normal distribution, data were analyzed by two-way ANOVA followed by Tukey’s test for repeated-measurement pair-wise comparison within the bacteria, and HIN bacteria for their effect on the amount and type of MMP-like activity, and three-way ANOVA followed by Tukey’s test for repeated-measurement pair-wise comparison within the bacteria, collagenase and HIN bacteria for their effect on the amount of hydroxyproline release (p <0.05).

Results

Generic and specific collagenolytic, MMP-like activities from E. faecalis:

E. faecalis showed generic and specific MMP-like activities with mean ± SD of 5.17 ± 0.47, 4.07 ± 0.14, 1.32 ± 0.24, 7.75 ± 0.88, and 5.98 ± 0.61 μM/3*10^6 CFU for generic MMP, MMP-1, -2, -8, and -9, respectively (Fig. 6.1). Generic and specific MMP-1, 8, and 9 activities were significantly higher than the HIN bacteria (P< 0.001), and the MMP 8-like activity was significantly higher than the other activities (p<0.05), followed by generic and MMP-9, MMP-1. The lowest activity was for MMP-2, not significantly
different from HIN. There was no significant difference between different MMPs-like activities for HIN (p>0.05).

**Human dentinal collagen matrix degradation:** The highest hydroxyproline release was for the collagenase positive control incubated groups. The mean hydroxyproline release for *E. faecalis* incubated groups was (1.8 ± 0.17 μg/50 μl) and was significantly higher (p<0.05) compared to HIN bacteria (0.61 ± 0.22 μg/50 μl) after 24 h of incubation (Fig. 6.2). SEM micrographs showed intact structure of the demineralized dentin organic matrix before exposure to any media (Fig. 6.3 A), minimal degree of degradation after heat inactivated *E. faecalis* incubation, smoother surface and more clear collagen fibrils network (Fig. 6.3 B). After exposure to overnight culture of *E. faecalis*, the specimens showed partial removal of the collagen fibril network and thinner fibers (Fig. 6.3 C). Collagenase type-I exposed groups showed a complete removal of the collagen fibril network from the intertubular dentin and inside the dentinal tubules in the same timeframe (Fig. 6.3 D).

**Discussion**

In this study, *E. faecalis* showed protease, MMP-like activities toward several MMP substrates relevant to known activities that degrade dentinal collagen [34, 48]. These measured MMP-like activities were in levels that degraded human tooth dentinal collagen matrix. These enzymes could contribute to the breakdown of the collagen component of dentin; undermining the durability of dentin bonding [417], contributing to increase in the deterioration of the sealer-tooth margins and explaining in part the pathogenicity of *E. faecalis* as a coronal invader [120]. These degradation processes could allow for bacterial biofilm proliferation and migration to periapical tissues [4], and could result in fracture of endodontically treated teeth [51], both affecting the success of root canal treatment.

Bacterial protease collagenolytic enzymes have been linked to many human diseases due to their ability to degrade native collagen [50], assist the spread of the pathogen into host tissues, contribute to the availability of amino acids for survival and growth, and facilitate toxins’ diffusion [418-420]. Clostridial collagenases were the first ones to be identified
and characterized and are the reference enzymes for comparison of newly discovered collagenolytic enzymes [421]. Bacterial collagenolytic proteases include some metalloproteases that cleave collagen in a similar manner to human MMPs [299]; therefore, it is not surprising to see that *E. faecalis* had activity toward the MMP substrates used in this study. MMPs have been widely studied for their important role in mammal development and human diseases [300, 301]. MMPs have been isolated from dentin, odontoblasts, pulp, periapical tissue [422], and saliva [423]. They are suggested to be responsible for the digestion of collagen fibrils exposed at the adhesive interface [424]. MMP family contains 23 members that are divided into six groups based on substrate specificity and homology including collagenases and gelatinases [424].

Collagenases (MMP-1, -8, -13), and MMP-2 (gelatinase A), can cleave native triple-helical type I, II, and III collagens; the collagen peptide structure determines both specific cleavage and binding sites for MMPs [425]. The denatured fragments are further degraded by gelatinases and other non-specific tissue proteinases [424]. Despite this classification, most MMPs can degrade several substrates with variable specificity [285]. MMP-1 and -13 are collagenase enzymes that can also degrade gelatin at a slow rate; MMP-2 and -9 are gelatinases that can degrade several types of collagen [285, 426]. MMP-1, -2, -8, and -9 showed to be involved in degrading dentinal collagen [34, 282, 302].

The activity of *E. faecalis* toward the MMP substrates in the measuring kits further suggest a similarity in action between the bacterial collagenase and human MMPs. Using the fluorometric MMP assay kits as a first diagnostic line was practical and relevant; fluorescence-based assays showed higher sensitivity, larger signal-to-noise windows, and requirement for very low volumes of reagents when compared to the colorimetric assays [427]. The bacterial collagenolytic metalloproteases include many families that cleave collagen chains at different sites, therefore even lack of response to MMP kits does not exclude the protease collagenase activity of the bacteria [299].
MMP-like activities of *E. faecalis* was found to be at least 50 times more than what was reported for dentinal and salivary MMPs activities [34, 302], accordingly the ability of the bacteria to degrade the dentinal collagen is expected to be much faster than the slow degradation reported for the host derived dentinal enzymes [34], and as such, contribute to the degradation of the matrix much more than the collagenolytic activities found for human dentin measured in the current study. The highest measured bacterial activity was toward MMP-8 substrate. MMP-8 was found to be the major collagenase in human dentin [282], its preference toward type I collagen as a substrate indicate major virulence factor that contributes to endodontic failure [428], since this is the major interstitial collagen type in the dentin organic matrix [429].

True degradative activity toward dentinal collagen was verified by the production of hydroxyproline using a demineralized dentin collagen model that has been used previously as a suitable representative model in collagen degradation studies [34, 297]. The amino acid sequence of collagen type-I contains glycines, and rich in hydroxyproline and proline [294]. Hydroxyproline is a collagen degradation product; its presence in the hydrolysate indicates the tissue-collagen concentration [295, 296], the stability, and resistance of collagen fibers to degradation by proteases [297, 298]. In the current study, dentinal samples incubated with either collagenase type I, or *E. faecalis* showed significantly higher release of hydroxyproline compared to HIN bacteria. Heat inactivation of bacteria is a technique used in bacterial enzymes studies to prepare a control group to which the active (non-heated) bacteria can be compared [403, 430]. In this study the heat inactivation of *E. faecalis* reduced the CFU, decreased the MMP-like activity, and reduced the hydroxyproline release of the dentinal samples significantly in comparison with the active bacteria, supporting the suggestion that the changes in MMP activity and dentinal collagen degradation are due to true enzymatic activity from the wild strain bacterial cells and are not due to non-specific and general effects, such as pH changes. The latter were verified to be similar for both parent and HIN bacteria.
SEM micrographs provide further support for the hypothesis that specific enzymes from the bacteria are responsible for the degradation of dentinal collagen observed in this study by showing an increase in collagen destruction by collagenase type I and the wild type strain, but not the HIN bacteria.

Makinen et al. [150], provided further indication of the ability of the bacteria to destruct the organic material of the demineralized dentin by showing the ability of gelatinase purified from *E. faecalis* to degrade soluble and insoluble collagens [150]. This protease activity could possibly explain the role *E. faecalis* and other pathogenic species in root canal refractory periapical infections by facilitating their migration from the root canal into the periapical lesion [15, 434]. However, the broad activity profiles of *E. faecalis* toward different MMPs found in the current study suggest that there is likely more than one specific enzyme that is involved in the degradation of dentinal collagen.

**Conclusions:** *E. faecalis* possesses collagenase protease activities that are capable of degrading dentinal collagen, thus could potentially compromise the sealer-dentin interface and tooth structure. Further studies are required to identify and characterize the specific proteolytic activity from the bacteria to allow for the development of anti-collagenolytic sealers and/or treatment that could be used to mitigate the effect of the bacteria.
Figures

**Figure 6.1:** Generic and specific MMP -1, -2, -8, and -9 MMP activity profiles of *E. faecalis*, and heat inactivated (HIN) *E. faecalis*. Values were normalized to buffer with the kit's substrate. The bacteria showed significant generic and specific MMP -1, -8, and -9 activity compared to HIN bacteria. Data are shown as mean ± SD (N=3/group). *E. faecalis* showed significant generic and specific -1, -8, and -9 MMP-like activities compared to HIN bacteria with the highest activity for MMP 8-like activity. There was no significant difference between different MMPs-like activities for HIN. Different letters indicate significant differences between groups (p<0.05).
Figure 6.2: Amount of hydroxyproline μg/ 50 μl after incubating the dentin samples for 24 h in either, E. faecalis in CDM (Chemically defined media), heat inactivated (HIN) E. faecalis, and Type I collagenase, results were normalized to readings from CDM incubated samples. Data are shown as mean ±SD (N=3). Different letters represent significant difference between groups. The highest hydroxyproline release was for the collagenase incubated group, followed by E. faecalis incubated group that was significantly higher (p<0.05) compared to HIN bacteria after 24 h of incubation.
Figure 6.3: Representative SEM microphotographs (10K X), showing the structure of the demineralized dentin collagen network and the dentinal tubules (DT). A) Sample was not exposed to any media after demineralization; the collagen fibers were almost intact. B) Minimal degree of degradation and smoother surface after HIN *E. faecalis* incubation. C) After exposure to overnight culture of *E. faecalis* the specimen showed partial destruction of the collagen fibril network compared to HIN, and thinner collagen fibers, the arrows shows the bacteria penetrating the dentinal tubules. D) Collagenase type-I exposed group showed a complete destruction of the collagen fibril network, with no more fibrillar structure can be noticed.
Chapter 7

Discussion and Conclusions

7.1 Summary

Some of the teeth receiving endodontic treatment do not heal [13], and require further root canal treatment that costs high burden on patients and the society. The cause of root canal treatment failure is microbial biofilm proliferation of the root canal treated teeth [42]. The first part of this study investigated the effect of simulated human salivary esterases (SHSE) on the biostability of the root canal sealer-dentin interface, and physical properties of root canal sealers. The second part explored the effect of *E. faecalis* esterases and collagenolytic-like activities on the degradation of methacrylate resins and root dentin. The findings showed that SHSE increased the sealer-dentin interfacial degradation, as demonstrated by increased bacterial biofilm proliferation within the sealer-dentin interface, and had an adverse effect on some of the physical properties of the root canal sealers. *E. faecalis* showed hydrolase, esterase-like and collagenase-like activities in levels that degraded methacrylate resins and dentinal collagen, respectively. Those hydrolase activities would likely facilitate the bacterial migration from the root canal into the periapical area, causing root canal treatment failure.

The study adopted a non-invasive chemostat based fermenter model that has been used previously by Roth et al. [52] to suspend root samples and cultivate bacterial biofilm, with the addition of using newly introduced sealers, and CLSM to be able to view the intact biofilm in thin section and to reconstruct these sections into a 3D structure for better understanding of the distribution of the bacterial cells. Live/dead ratio was also used to give an indication of the antimicrobial activities of different root canal sealers over time. Lastly and one of the most important additions to our project is the use of SHSE as pre-incubation media trying to simulate to some extent the *in vivo* oral conditions.
Finer & Santerre showed that saliva possesses CE and PCE-like activities [384], and those activities were able to degrade the methacrylate resin materials [388], accelerate the degradation of the resin-coronal dentin interface, increase the interfacial microbial biofilm proliferation [43, 53], and decrease the bond strength between resin and dentin [58]. The current study is the first to introduce the potential effect of the salivary esterases on the sealer-root dentin interfacial integrity measured by the depth of bacterial biofilm proliferation, and the effect on root canal sealer physical properties. Methacrylate resins have the potential to be used as a root canal sealers, but their use nowadays in root canal is mainly limited to post cementation [60], and restoration of endodontically treated teeth [35], while epoxy resin (ER) and bioceramic sealer (BC) are more commonly used as a root canal sealers [52]. The adverse effect of SHSE on the marginal integrity of the methacrylate resin-dentin interface was expected due to the presence of ester linkages in the monomers of the methacrylate resin materials [371, 377], while no effect of the enzyme was expected on ER, and BC-dentin interfaces. The results confirmed the first part of the hypothesis, and disagreed with the second part as SHSE increased the interfacial biofilm proliferation for BC groups.

The results of interfacial bacterial biofilm proliferation were interesting and worth further investigations on the possible mechanism of how could SHSE affect some critical physicochemical properties of the three dental materials used in the bacterial biofilm proliferation study. Accordingly, surface microhardness, compressive strength, dimensional change, and solubility/weight loss of BC, ER, and RC were tested after exposure to SHSE or PBS for up to 4 weeks. The results showed that SHSE affected some of the physical properties of BC and RC and this could be explained probably by the effect of the enzyme on BC hydration setting process [52], and due to hydrolysis of the ester-links within RC [187, 188]. ER sealer showed the highest stability in both PBS and SHSE, and this stability was matching other reported results [44].

Bourbia et al. [13] found that the cariogenic bacteria S. mutans has esterase-like activity in levels that enable it to degrade dental methacrylate resins. The root canal infection is complex and is the result of infection by multispecies biofilms [15]. E. faecalis has been
detected frequently from failed root canal cases [120], it is able to survive harsh environment, and is considered as a secondary coronal invader [116]. *E. faecalis* is not the only species that participates in root canal failure and in some failed cases it might not be present [42]. Nevertheless, it is still relevant to endodontic research and has been used as an indicator to assess the sealer-dentin interfacial integrity [134], antimicrobial activity of the sealers [288], and therefore can be employed to develop experimental models to investigate other species or multispecies biofilms. In this study, the hypothesis was that *E. faecalis* has esterase-like activities that facilitate the bacterial proliferation through the coronal methacrylate resin restorations and post resin sealer cements contributing to root canal treatment failure. The results of the study confirmed the hypothesis and showed that *E. faecalis* possesses esterase-like activities that will accelerate its proliferation through the resin-dentin interface by hydrolyzing the restoration part of the interface.

Another important hydrolyzing activity that might affect the dentinal part of the sealer-dentin interface is collagenase activity. Collagen is the major protein in the dentinal matrix [51], and it is important to maintain the structural, mechanical, and functional integrity of the root dentin [59], and preserve the rein-dentin bond [150]. Makinen P.L et al. [150] showed that the gelatinase purified from *E. faecalis* was able to degrade soluble and insoluble collagens [33, 42]. In this part of our study we hypothesized that *E. faecalis* possesses collagenase-like activities that degrade the dentinal collagen matrix of teeth samples and might subsequently facilitate the bacterial proliferation as a coronal invader from the crown to the apex of the root participating in the periapical infections. The results of the study confirmed the hypothesis and showed quantitatively and qualitatively the ability of *E. faecalis* to degrade the dentinal collagen matrix.

### 7.1.1 Conclusions

- The results of previous studies were important since they showed that the clinical scenario is very different from the *in vitro* conditions, and testing the dental materials by aging them in distilled H$_2$O or buffers cannot be regarded as relevant to the clinical
condition. The more the *in vitro* condition simulates the oral cavity conditions, the more likely that the results will reflect intraoral conditions.

- The enzymatic activities of the bacteria are controlled by many factors in the oral cavity. These include the pH, other oral enzymes, the dental materials biodegradation by-products and the bacteria’s growth mode.

- The experimental techniques used in this project can be used to investigate newly introduced sealers and other endodontic pathogens in their biofilm growth phase.

- The development of anti-collagenolytic, and esterase resistant sealers and/or treatment could be used to mitigate the effect of the salivary and bacterial enzymes.
7.2 Discussion

It is critical to assess the factors that could facilitate the microbial proliferation into the root canal system as these could contribute to treatment failure. Knowledge of these factors would help to develop methods to counteract their effects. The current study assessed the effect of simulated human salivary esterase activities (SHSE) on the integrity of the sealer-dentin interfacial margins and on some of the physical properties of root canals sealers. It investigated also the effect of esterase and collagenolytic-like activities of *E. faecalis* on the degradation of the dental methacrylate resins, and the structural stability of the dentinal collagen matrix. The results showed that SHSE increased the sealer-dentin interfacial degradation as measured by the increased bacterial biofilm proliferation, and had an adverse effect on some of the physical properties of the root canal sealers. *E. faecalis* showed hydrolase; esterase-like and collagenase-like activities in levels that degraded methacrylate composites and dentinal collagen, respectively.

7.2.1 The effect of SHSE on the biostability of sealer-dentin interfacial margins

Assessing the sealer-dentin biostability after aging for one year in SHSE or PBS, followed by challenging the samples with *E. faecalis* biofilm resulted in bacterial biofilm proliferation in all samples that was dependent on sealer type, pre-incubation period and condition. SHSE incubation accelerated interfacial degradation, indicated by increased interfacial biofilm proliferation of BC, TE, and SE at some time points, and increased the live/dead ratio for all groups at different time points.

The use of CBBF allowed for continuous controlled flow of BHI, simulating pathogenic oral conditions to cultivate *E. faecalis* biofilm [15]. *E. faecalis* was used in the bacterial proliferation study as a biological marker to assess the root canal sealer-dentin interface stability. It is a facultative anaerobic coccus that is commonly detected in asymptomatic, persistent endodontic infections [15, 68, 117, 122]. The survival of *E. faecalis* after endodontic therapy has been attributed to many virulence factors [13], one of the most important factors is the ability to form biofilm [42].
Samples were first aged (pre-incubated) individually in sterile glass vials containing either PBS or SHSE for up to 360 days, followed by incubation in CBBF to cultivate *E. faecalis* for 3 days. The two steps (pre-incubation and incubation) were separated and the time lines were chosen for the following reasons:

1. It is very demanding to run the CBBF for up to 360 days, with a high risk of contamination of the samples, and the bacteria will cover the whole sample making it difficult to diagnose the interface and be able to count the viable and dead bacteria.

2. There is limited space within the CBBF to accommodate all specimens from the different groups and time frame for such a long period, and it is impossible to analyze all the samples under CLSM at the same time while maintaining the viability of the bacteria and the fluorescence of the live/dead stain.

3. Previously a 7-days CBBF incubation period was used [435], the current study modified the incubation period to 3 days instead. This modification was based on the following:

   A) A pilot study that found no significant difference in the depth of interfacial bacterial proliferation between 7 and 3 days. However, it was hard to count the live / dead bacterial cells for the 7 days incubation groups.

   B) Seneviratne et al. [436] showed that *E. faecalis* biofilms reached maturation at 3 days when a nutrient-rich medium containing glucose was used.

The specimens were prepared from the coronal third of the roots trying to standardize the samples as much as possible by eliminating the more complex and branched apical part of the root [64]. The coronal third is usually the first part of the root to be challenged by the bacteria invading the root canal [76]. *E. faecalis* is a coronal invader that gains entrance to the root canal through the coronal part of the tooth and succeeds in colonizing the canal [23-25, 343].

ER was selected in this study as it is considered the gold standard in comparative studies of root canal sealers due to its favorable physical, biological and antimicrobial properties.
BC sealer is a newly introduced sealer that showed tolerance to moisture and small amounts of blood, good antimicrobial, sealing, and mechanical properties [345]. RC is a methacrylate resin polymer that is mainly used nowadays in root canals for post cementation [346, 347], and restoration of endodontically treated teeth [52], but can be potentially used as a root canal sealer. It is vulnerable to hydrolysis; salivary [44], and bacterial [33, 43] esterase can catalyze this process and accelerate the degradation rate of the resin-dentin interface [348]. Either TE or SE adhesive systems are used to bond RC to tooth structure. In the absence of proper coronal seal saliva and microbes can cross the root canal affecting the treatment success [52]. Therefore, it is imperative to investigate the effect of the sealer-dentin interface in an environment where salivary enzymes are present and explore any detrimental effect on their performance.

SHSE is a mixture of CE and PCE that was chosen for its potential to affect the sealer-dentin interfacial stability. Saliva has been shown to contain both CE and PCE-like esterases activities [52], both esterases act synergistically to increase the biodegradation of methacrylate resin dental composites and adhesives [43, 53], decrease their bond to dentin [33], and increase the bacterial biofilm proliferation between the adhesive-dentin interfaces [173].

CLSM was used as a non-destructive method to determine the true architecture of plaque and the location of selected bacteria within the biofilm [33, 83]. This is a significant advantage to examine biofilms in situ without the limitations encountered with other methods, such as SEM [83, 174]. The use of CLSM requires that the organisms in the biofilms be stained with fluorescent stains. Using a suite of such stains allows the biofilm researcher to quantify all the cells and determine which ones are viable [349], and this could be used as an indication of the antimicrobial activity of the materials [351].

Comparing the four test materials, BC maintained the highest antimicrobial activity, while the lowest activity was for the methacrylate resin groups that had no antimicrobial advantage over the other sealers [352]. Increasing the number of live bacteria in some of SHSE pre-incubated groups could be explained by possible interactions between the protein and the bacteria [42], as well as the increase in interfacial gap with time [351,
ER and BC showed reduction in their antimicrobial activity with time, this result corresponds with many other studies that showed that most sealers lose their antibacterial activity over time [52].

The depth of bacterial biofilm proliferation varied between the four material groups at different time points and incubation media. At zero time all materials showed comparable sealing ability that deteriorated with time to end up after 360 days having the best sealing ability for BC and ER in SHSE, and for BC in PBS.

Methacrylate resin materials-dentin interfaces were the most susceptible to interfacial biofilm bacterial proliferation; SHSE incubation increased this susceptibility probably due to hydrolysis of the ester-links within the resin materials [33], and increasing the shrinkage of the material that had been shown after assessing its dimensional change in SHSE, resulting in interfacial degradation and bacterial biofilm proliferation [15, 16, 68].

7.2.1.1 Conclusions

ER was more stable in solutions and salivary enzymes compared to other sealers. BC sealer showed the best sealing ability and antimicrobial activity when compared to other sealers after 360 days of aging in saline, while its sealing ability was comparable to ER after 360 days of SHSE exposure. SHSE had an effect on the bacterial biofilm proliferation depth and live/dead ratio, and therefore might compromise the sealer-dentin interface. This shows the importance of using incubation media with enzymatic activities similarly to those that exist in human saliva rather than the common use of buffers or H₂O as incubation media for the assessment of aging on the material properties.
7.2.2 The effect of SHSE on the physical properties of root canal sealers

The results of the bacterial proliferation study were interesting, and worth further investigations to elucidate the mechanisms in which SHSE affected the physical properties and sealing ability of RC and BC but not the ER, and to answer these questions we assessed selected root canal sealers’ physical properties that are related to their clinical performance when in contact with PBS or SHSE for up to 28 days, using experimental techniques that have been recommended for standard testing of endodontic materials [366]. The results showed that SHSE affected the microhardness, weight loss, and dimensional change of BC and RC, while ER physical properties were stable in both PBS and SHSE.

Surface microhardness is an indicator of the setting process of the material [367], and a significant parameter to measure the root canal sealer’s ability to resist applied occlusal stresses within the canal [368, 369]. Hardness can be used as an indicator of the hydration process of hydraulic cements [370], and the polymerization extension of dental resins [41, 371, 372]. In this study, the surface microhardness of BC, ER, and RC were evaluated after incubation in PBS or SHSE for 7 and 28 days. The results demonstrated that SHSE exposure had a detrimental effect on the surface microhardness of BC and RC. The hydration process and the crystalline structure of BC are affected by the environmental conditions and this could have an adverse effect on the surface hardness of the cement [350, 373]; the proteins in SHSE might be adsorbed to the cement reducing the water uptake, thus delaying the setting and reducing the hardness of BC [31, 374]. Methacrylate resins are vulnerable to hydrolysis due to the presence of ester linkages within its monomers [44, 52, 335]; the hydrolysis process can be further catalyzed by the presence of esterase enzymatic activity in saliva and bacteria [52]. RC showed reduced microhardness, that could be explained by water sorption and hydrolytic reaction that is catalyzed by esterases in SHSE [52]. It is expected that the hydrolysis process in the oral cavity will be further accelerated for this material due to the chewing forces and the presence of food [375].
In addition to the testing of surface microhardness, the compressive strength of the materials was examined. Higher compressive strength values make root canal sealers more durable and resistant to the displacement of cones during post and core placement [376]. Clinically accelerated setting time is a desirable property of endodontic materials [376]. Compressive strength is used to monitor the setting process of the root canal sealers, and it is a greater indication of the sealer’s strength than the setting time [375], as some materials’ strength continues to increase when the setting time is recorded [377].

According to the results of this study, the compressive strength of the three materials was not affected by SHSE incubation. BC strength had increased with time and this was reported by another study [378], and could be explained by continuing hydration and crystallization of the calcium silicate-based cements for up to 4 weeks [35, 378]. Microhardness values for BC were lower than what was reported for other studies [35], and this could be explained by the need to immerse one side of the samples in water during setting in order to get hard samples, and in this process it was hard to standardize the amount of the absorbed water under in vitro conditions and could have adversely affected the compressive strength [379]. RC compressive strength was comparable to the values that were reported previously [365]. Since the minimum compressive strength that is required for root canal sealers is not reported in the literature, the higher compressive strength of RC does not necessarily indicate a better clinical performance of this material compared to ER and BC.

Excessive shrinkage and expansion are not desirable properties for a root canal filling material. Shrinkage of non-bonded sealers produces pathways for bacteria and their product [380], and could cause internal stresses, while expansion of the material may elicit cracks in the root [363]. The dimensional change of BC, RC and ER was in agreement with ISO 6876/2012 specifications, which recommends that dimensional change for root canal sealers should not exceed 1.0% shrinkage or 0.1% expansion. BC showed slight expansion in PBS, while incubation with SHSE for 7 days reduced the expansion. This reduction in expansion could be explained by the enzyme penetration into the porosities within the sealer and therefore reducing the water uptake and expansion [381]. This slight expansion may contribute to superior sealing ability. Thus,
further tests are required to ascertain if BC effectively seals root canals without increasing the risk of development of cracks or root fracture. RC and ER showed shrinkage over the 28 days in both incubation media; the shrinkage was higher for RC after 7 days in SHSE compared to PBS. RC and ER are polymer-based, therefore polymerization shrinkage is expected upon material setting [382]. RC is a combination of inorganic particles that are coated by a coupling agent, dispersed in an organic methacrylate resinous matrix. Non-polymerized monomers are arranged and kept together by van der Walls forces. During the polymerization these forces are substituted by covalent bonds, and consequently volumetric shrinkage occurs [383].

High solubility of root canal sealers is undesirable and has been linked to the irritation of periapical tissues [187], and may increase the sealer-dentin interfacial bacterial leakage over time [322, 323]. All tested materials showed solubility within the limit allowed in the ISO 6876/2012 recommendations (3% mass fraction). Monomers and degradation by-products from the materials could affect gene expression and protein of the bacteria [210]. A more porous matrix may be produced when the sealer sets in the presence of moisture in wet canals, which, in turn, may result in increased leaching of tissue-irritating substances from the set sealer [368]. SHSE increased the solubility of BC at 28 days and this could be explained by the improper crystallization of the cement that makes it more vulnerable to hydrolysis and/or dissolution by the media [52]. The increase of RC solubility following 7 days of incubation in SHSE could be a result of ester links hydrolysis and release of byproducts [31, 33, 43, 52, 53, 335, 384, 385]. Many studies have shown the ability of esterases to degrade dental composites indicated by the release of the biodegradation product Bis-hydroxy-propoxy-phenyl-propane (BisHPPP), adversely affecting the bond between composite and tooth structure, and increasing the depth of bacterial proliferation between the tooth-composite interface [33]. The process is slower in the absence of esterases, but after 28 days, both PBS and SHSE contribute similarly to the weight loss of RC. The number of readily accessible ester linkages within the resin matrix gradually declines over time [64] modulating the effect of SHSE on weight loss.
ER physical properties were the most stable in moisture and salivary esterases, and this could be explained by its hydrophobic properties and lack of ester bonds in its structure. When compared to BC and RC, ER showed the lowest solubility and dimensional change, with microhardness that is almost comparable to BC and less than that of RC, and a compressive strength midway between the two materials.

BC sealer expanded and lost weight after exposure to SHSE and PBS, these findings could help in explaining the interfacial bacterial proliferation results; the continued expansion of the material after setting could explain the problem encountered with imaging the 30-days group, that later disappeared because of the material solubility. After 360 days of pre-incubation in saline BC was the least prone to bacterial proliferation probably due to the material expansion, in addition to the absence of degradable monomers in its structure.

7.2.2.1 Conclusions

Within the limitations of this laboratory study, the result showed variability of the physical properties between the three sealer materials. ER physical properties were not affected by moisture or enzyme exposure. This material had the least weight loss and dimensional change, and acceptable microhardness and compressive strength. RC had the highest microhardness and compressive strength, but it has the greatest shrinkage, and some of its properties were adversely affected by the presence of SHSE. BC was the only material that slightly expanded upon aging, but the higher dissolution rate and susceptibility to the presence of SHSE could impact its clinical performance. In general, materials used in the current study showed solubility and dimensional changes within the limitations of ISO (6876/2012). This highlights the importance of using incubation media with enzymatic activities similarly to those that exist in human saliva rather than the more common use buffers or water as incubation media for the assessment of aging on the material properties. As some physical properties continued to change over the 28 days incubation period, longer periods of incubation might be needed to have a better idea of the materials performance in both PBS and SHSE.
7.2.3 Degradative hydrolase activities from *E. faecalis*

After confirming that salivary esterases had significant degradative activities on the sealer-dentin interface, presented by bacterial biofilm proliferation, it was interesting to see if *E. faecalis* (as it is commonly detected in failed root canal cases) possess any degradative activities that could accelerate the interfacial degradation. Therefore, the esterase-like and collagenase-like activities of *E. faecalis* were assessed.

Bacterial infection caused by coronal leakage is a significant contributor to endodontic failure [389]. Bacterial pathogenicity includes secretion of enzymes that causes tissue damage [424]. Esterases and proteases are classified as hydrolases; they enzymatically add water across ester or peptide bonds [390]. Bacterial esterases have been linked to virulence and pathogenicity of some bacteria [391]. *Mycobacterium tuberculosis* carboxylesterase hydrolyzed the free esters in the local environment and accordingly could be a source of fatty acids to be used nutritionally by the bacteria [392]. Esterase from Group A Streptococcus was found to contribute to severe invasive infections [437], and to reduce neutrophil infiltration to the site of infection by hydrolyzing the ester bond of platelet-activating factor [44]. More recently, esterase activity from the cariogenic bacteria *Streptococcus mutans* found to hydrolyze resin composite and adhesives and therefore has the potential to degrade the restoration-tooth interface [50, 299]. Bacterial collagenases are metalloproteinases and serine proteases that are involved in the degradation of the extracellular matrices of animal cells, due to their ability to digest native collagen. Collagenase enzymes are important virulence factors in a variety of pathogenic bacteria [63], and may contribute to the degradation of collagen fibrils and deterioration of structural integrity in root dentin with time [52].

7.2.3.1 Esterase-like activities of *E. faecalis*

The results of the esterase activity study supported the hypothesis that *E. faecalis* ATCC29212 contains esterase-like activity at levels capable of degrading cured methacrylate resin composites and adhesives. This finding could explain part of *E. faecalis* abilities as a secondary coronal invader to penetrate through the interface
between coronal methacrylate restorations, and post cements entering the root canal causing secondary and periapical infections. The bacterial enzymes showed higher activity toward degrading TE, followed by RC, and the least activity was toward SE.

Human saliva [44] and S. mutans [52] have been shown to hydrolyze composite resins and adhesives. Human salivary esterases have shown activity toward nitrophenyl esters and BTC [44], while S. mutans had activity towards the nitrophenyl esters, but not BTC. Strains of S. mutans showed different activity profiles toward the nitrophenyl substrates [43, 275]. In the current study, E. faecalis expressed minimal activity toward BTC and a higher activity toward nitrophenyl esters in similar levels to several strains of S. mutans that were shown previously to degrade resin composites and adhesives [31, 42-44, 53]. E. faecalis activity levels and profiles were closer to that of S. mutans than that of salivary esterases, specifically JH1005, LT11, UA140, and BM71 strains that are linked to dental caries with preference toward p-NPA more than p-NPB.

Resin composite (Z250, 3M), total etch adhesive (Scotchbond™ Multipurpose, 3M), and self-etch adhesive (Adper™ Easybond, 3M) were selected in the current study as they are commonly used materials in many dental practices; and since these materials were extensively investigated, there is good baseline data regarding their biostability [387]. These materials are good representatives of TE, SE and RC group of materials so the results of this study could be generalized to similar materials from each group. All three materials are BisGMA-based; making the analysis of the degradation and comparison feasible and standardized [33, 43, 44, 385, 387].

BisHPPP is a biodegradation by-product of BisGMA, a universal monomer present in most adhesives and composite materials. BisHPPP was chosen for analyses since it is a good marker of true resin biodegradation due to the hydrophobic nature of its precursor [44]. E. faecalis significantly increased the amount of BisHPPP released for RC and TE adhesive after 30 and/or 14 days of incubation, while there was significantly lesser effect on the SE material compared to the control. The total amount of BisHPPP released in the samples incubated with E. faecalis over 30 days was significantly higher in TE adhesive
compared to RC and SE adhesive. The percent composition of BisGMA of total-etch adhesive, self-etch adhesive and composite are 60-70, 15-25, and 5-10% of the total mass of the materials, respectively [3]. Since the amount of BisHPPP released was not correlated with the BisGMA content per material, and since all materials were similar in their surface area, volume, and degree of vinyl group conversion [393], this difference could have arisen only from the inherent difference in chemical composition of the materials and their interaction with the specific activities of *E. faecalis*.

Susceptibility of the polymers to microbial degradation generally depends on enzyme availability, enzyme specificity for a polymer, availability of a site in that polymer for enzyme attack, and the presence of coenzyme, if required [44]. Different biodegradation profiles were found for *S. mutans* UA159 vs. *E. faecalis* ATCC 29212. *S. mutans* UA159 showed different preferences than *E. faecalis* ATCC 29212 toward the nitrophenyl substrates, with preferences toward pNPB compared to pNPA, as well as differences in the degradation of the restorative materials, RC, TE and SE adhesives [394, 395]. This further suggests that specific enzymatic activity from each species is responsible for the observed activity profiles and degradative activities. Variations in the enzymatic activities are expected due to the difference in the potential esterase genes between the different strains [44, 396-398].

Following 30-days of incubation, SEM analysis showed an increase in surface roughness for all incubated samples compared to the non-incubated samples, indicating the degradation of all materials. This observation corroborates previous studies that showed surface degradation and changes in surface-topography of polymer-based restorative materials by bacteria [44]. However, except for Bourbia et al. [399], these studies provided only observation of surface changes without characterization of possible degradative activities from the bacteria. The current study is the first to characterize esterase activities by *E. faecalis* that degrade methacrylate-based restorative materials.

Esterase-like activities were different for each growth phase of the bacteria, with the lag phase having the lowest enzymatic activity followed by the stationary phase and the
highest values were for the log phase. This could be explained by the physiology of each phase; the lag phase allows the adaptation required for bacterial cells to begin to exploit new environmental conditions [400], and the cells are metabolically less active than the other phases [399]. In the log phase cell division proceeds at a constant rate and it is considered as the most active metabolically [401], while the stationary phase starts when conditions become unfavorable for growth and bacteria stop replicating and reducing its metabolism. The transition between the log and stationary phases involves a period of unbalanced growth during which the various cellular components are synthesized at unequal rates. Consequently, the differences in the metabolic activities from those physiological phases could have led to the difference in the enzymatic activity noticed in the current study [402].

The heat inactivation (HIN) process affects the structural and physiological properties of every cellular component of the bacteria (membrane, enzymes and proteins, DNA, and RNA) [403, 404]; Therefore, this process is useful to establish a group of inactive bacterial suspensions to act as a control to which the live bacterial suspensions can be compared [405]. In the current study HIN *E. faecalis* showed a significant decrease in the esterase-like activity of the bacteria. Since the pH and the growth conditions were similar for the two bacterial cultures, it could be hypothesized that the measured activity is due to true enzymatic activity from the bacteria and not due to other factors, such as low pH, or the incubation media.

The supernatant had a significantly less measured esterase activity than the bacterial suspensions under the conditions of the current study. However, the absence of the enzymatic activity of the supernatant does not exclude the possibility that the enzyme was released, as the bacteria utilize several methods to invade mammalian hosts, damage tissue sites, and thwart the immune system from responding [406]. These include transport of proteins from the cytoplasm across the inner membrane into the bacterial supernatant or onto the surface of the bacterial cell [408]. The release of cell contents following bacterial cell death, nutrients and enzymes, such as those involved in the process of hydrolysis of dental materials will contribute to the continued survival of the
biofilm population as a whole [33]. Overall, the fact that the bacteria hydrolyzed the resinous materials in the current study confirms that enzymes were either released or attached to the cell wall, though the exact mechanism and identity of the enzyme requires further investigation.

Kermanshahi et al. [15] showed that exposure of dentin-composite restorations to salivary esterase-like activity resulted in the formation of gaps that were infiltrated and colonized by bacterial biofilms. *E. faecalis* could contribute to the deterioration of the resin-dentin interface when present within the restoration-tooth marginal gap by releasing both protease collagenase enzyme (chapter 6) and esterases, affecting the hybrid layer, tooth and composite, and potentially compromising the integrity of the margins, reducing the longevity of the restoration, increasing the bacterial proliferation into the root canal system, and contributing to the failure of root canal treatment [31, 241, 409]. Increasing the esterase resistance of adhesives and of methacrylate composites could potentially improve the materials biochemical stability, and extend the longevity of resin-dentin bonds, therefore increasing the lifespan of the dental resin restorations [417].

### 7.2.3.1.1 Conclusion

*E. faecalis* possesses hydrolases, esterase-like activities that carry the potential to compromise the sealer-dentin interface. The overall activity patterns of *E. faecalis* suggest that it could be a contributor to acetate-like dependent esterase activities of saliva and less to the butyrate-like dependent esterases that are characteristic of human salivary esterase activity. The extent of dental resin composites and adhesives degradation by *E. faecalis* was material dependent and material chemistry was the most important factor in determining the material’s biochemical stability. When present within the confined space of the sealer-tooth or cement-tooth marginal interface, *E. faecalis* could contribute to the deterioration of the resin-dentin interface and consequently accelerate its proliferation within the root canal leading to compromised treatment success.
7.2.3.2 Collagenolytic-like activities of \textit{E. faecalis}

\textit{E. faecalis} showed protease, MMP-like activities toward several MMP substrates. These measured protease activities were in levels that degraded human tooth dentinal collagen matrix. These enzymes may contribute to the breakdown of the collagen component of dentin, undermining the durability of dentin bonding [120], contributing to increase in the deterioration of the sealer-tooth interface, explaining part of \textit{E. faecalis} pathogenicity as a coronal invader [4], allowing for bacterial biofilm proliferation and migration to periapical tissues [51], and might result in weakening of endodontically treated teeth [50], affecting the success of root canal treatment.

Bacterial protease collagenolytic enzymes have been linked to many human diseases due to their ability to degrade native collagen [418-420], assist the spread of the pathogen into host tissues, contribute to the availability of amino acids for survival and growth, and facilitate toxins’ diffusion [421]. Clostridial collagenases were the first ones to be identified and characterized and are the reference enzymes for comparison of newly discovered collagenolytic enzymes [299]. Bacterial collagenolytic proteases include some metalloproteases that cleave collagen in a similar manner to human MMPs [300, 301]. Therefore, it is not surprising to see that \textit{E. faecalis} had activity toward the MMP substrates used in this study. MMPs have been widely studied for their important role in mammal development and human diseases [422]. MMPs have been isolated from dentin, odontoblasts, pulp, periapical tissue [423], and saliva [424]. They are suggested to be responsible for the digestion of collagen fibrils exposed at the adhesive interface [424]. MMP family contains 23 members that are divided into six groups based on substrate specificity and homology including collagenases and gelatinases [425].

Collagenases (MMP-1, -8, -13), and MMP-2 (gelatinase A), can cleave native triple-helical type I, II, and III collagens; the collagen peptide structure determines both specific cleavage and binding sites for MMPs [424]. The denatured fragments are further degraded by gelatinases and other non-specific tissue proteinases [285]. Despite this classification, most MMPs can degrade several substrates with variable specificity [285, 426]. MMP-1 and -13 are collagenase enzymes that can also degrade gelatin at a slow
rate; MMP-2 and -9 are gelatinases that can degrade several types of collagen [34, 282, 302]. MMP-1, -2, -8, and -9 showed to be involved in degrading dentinal collagen [427].

The activity of *E. faecalis* toward the MMP substrates in the measuring kits further suggest a similarity in action between the bacterial collagenase and human MMPs. Using the fluorometric MMP assay kits as a first diagnostic line was practical and relevant; fluorescence-based assays showed higher sensitivity, larger signal-to-noise windows, and requirement for very low volumes of reagents in comparable to the colorimetric assays [299]. The bacterial collagenolytic metalloproteases include many families that cleave collagen chains at different sites, therefore even lack of response to MMP kits does not exclude the protease collagenase activity of the bacteria [34, 302].

MMP-like activities of *E. faecalis* were found to be at least 50 times more than what was reported for dentinal and salivary MMPs activities [34], accordingly the ability of the bacteria to degrade the dentinal collagen is expected to be much faster than the slow degradation reported for the host derived dentinal enzymes [282], and as such, contribute to the degradation of the matrix much more than the collag enolytic activities found for human dentin measured in the current study. The highest measured bacterial activity was toward MMP-8 substrate. MMP-8 was found to be the major collagenase in human dentin [428], its preference toward type I collagen as a substrate indicates major virulence factor that contributes to endodontic failure [429], since this is the major interstitial collagen type in the dentin organic matrix [34, 297].

True degradative activity toward dentinal collagen was verified by the production of hydroxyproline using a demineralized dentin collagen model that has been used previously as a suitable representative model in collagen degradation studies [294]. Collagen type-I chains are characterized by a repetitive Gly-X-Y amino acid motif where X and Y positions often correspond to proline and hydroxyproline, respectively [295, 296]. Hydroxyproline is a collagen degradation product and its concentration in the solution indicates the degree of collagen degradation [297, 298], and the stability of collagen fibers [431]. In the current study, dentinal samples incubated with either
collagenase type I, or *E. faecalis* showed significantly higher release of hydroxyproline compared to HIN bacteria.

The heat inactivation of *E. faecalis* reduced the CFU, decreased the MMP-like activity, and reduced the hydroxyproline release of the dentinal samples significantly in comparison with the active bacteria, supporting the suggestion that the changes in MMP activity and dentinal collagen degradation are due to enzymatic activity from the active bacterial cells and are not due to non-specific and general effects, such as pH changes. The latter were verified to be similar for both parent and HIN bacteria.

SEM micrographs provide further support for the hypothesis that specific enzymes from the bacteria caused the degradation of dentinal collagen observed in this study by showing an increase in collagen destruction by collagenase type I and the wild type strain, but not the HIN bacteria. The less clear network of collagen fibrils in the non-exposed samples could be due to the existence of non-collagenous proteins in the dentin’s organic matrix that are bound to the collagen fibers [432]. The acidity of the HIN suspension could have an effect on the stability of the non-collagenous proteins [433], exposing the collagenous fibers that might be more resistant to low pH as native collagen tissue possesses significant strength [150].

Makinen et al. [150] provided further indication of the ability of the bacteria to destruct the organic material of the demineralized dentin by showing the ability of gelatinase purified from *E. faecalis* to degrade soluble and insoluble collagens [15, 434]. This protease activity could possibly explain the role *E. faecalis* and other pathogenic species in root canal refractory periapical infections by facilitating their migration from the root canal into the periapical lesion [438]. However, the broad activity profiles of *E. faecalis* toward different MMPs found in the current study suggest that there is likely more than one specific enzyme that is involved in the degradation of dentinal collagen.
7.2.3.2.1 Conclusions

*E. faecalis* protease, collagenase-like activities were capable of degrading dentinal collagen, thus could potentially compromise the sealer-dentin interface and tooth structure, regardless of the sealer type and properties. The broad activity profiles of *E. faecalis* toward different MMPs substrates suggest that there is likely more than one specific enzyme that is involved in the degradation of dentinal collagen.
7.3 Conclusions

Within the limitations of these in vitro studies it can be conclude that:

1. In the results of the interfacial bacterial proliferation study; BC sealer showed the best sealing ability and antimicrobial activity when compared to other sealers after 360 days of aging in PBS, while its sealing ability was comparable to ER after 360 days of SHSE exposure. SHSE had an effect on the bacterial biofilm proliferation depth and live/dead ratio, and therefore might compromise the sealer-dentin interface.

2. Different root canal sealers have variable physical properties. However, all the materials used in the current study showed solubility and dimensional changes within the limitations of ISO (6876/2012). Between the three tested materials (BC, ER, RC), ER was the most stable to moisture and enzyme exposure. ER also showed the least weight loss and dimensional changes, and had acceptable microhardness and compressive strength. RC had the highest microhardness and compressive strength, but it has the greatest shrinkage, and some of its properties were adversely affected by the presence of SHSE. BC was the only material that slightly expanded upon aging, but the higher dissolution rate and susceptibility to the presence of SHSE could impact its clinical performance. This highlights the importance of using incubation media with enzymatic activities similar to those that exist in human saliva rather than the common use of buffers or H2O as incubation media for the assessment of aging on the material properties.

3. E. faecalis possesses hydrolases, esterase-like activities that carry the potential to compromise the sealer-dentin interface. The overall activity patterns of E. faecalis suggest that it could be a contributor to acetate-like dependent esterase activities of saliva and less to the butyrate-like dependent esterases that are characteristic of human salivary esterase activity. The extent of dental methacrylate resin degradation by E. faecalis was material dependent, and material chemistry was the most important factor in determining the material’s biochemical stability. When present within the confined space of the sealer-tooth or cement-tooth marginal interface, E. faecalis
could contribute to the deterioration of the resin-dentin interface and consequently accelerate its proliferation within the root canal, leading to compromised root canal treatment success.

4. *E. faecalis* protease, collagenase-like activities were capable of degrading dentinal collagen, thus could potentially compromise the sealer-dentin interface and tooth structure, regardless of the sealer type and properties. The broad activity profiles of *E. faecalis* toward different MMPs substrates suggest that there is likely more than one specific enzyme that is involved in the degradation of dentinal collagen.
Chapter 8

Future directions, and recommendations

8.1 Future directions

- Identification and verification of specific genes of esterase and protease activities in *E. faecalis* that are responsible for dental resin composite and adhesive and dentinal organic matrix degradation.

- Exploration of the interfacial microbial biofilm proliferation and the degradative activities using multispecies biofilm bacterial model, as it simulates the oral cavity and bacterial genotypic and phenotypic expression in a better way compared to monospecies system.

- Exploration of the impact that *E. faecalis* and other endodontic pathogens may have on the mechanical properties of root canal sealers.

- As some physical properties continued to change over the 28 days incubation period, longer periods of incubation might be needed to have a better idea of the materials’ performance in both PBS and SHSE.

8.2 Recommendations

- The methods used in this study to assess the interfacial microbial bacterial biofilm proliferation proved to be valuable and can be used to assess newly developed sealers before their introduction to the market.

- The methods used in this study to assess the collagenase and esterase-like degradative activities were sensitive and specific to identify relevant degradative activities of *E. faecalis*, and therefore they could be used to assess the degradative activities from other endodontic pathogens.

- The development of esterase resistant, and anti-collagenolytic sealers and/or treatment could be used to mitigate the effect of the bacteria and salivary enzymes.
- The use of media with enzymatic activities similar to those that exist in human saliva and/or oral bacteria rather than the common use of buffers or water as incubation media for the assessment of aging on the material properties is considered more clinically relevant.
Chapter 9

Supplementary data

9.1 Supplementary data for the bacterial proliferation study

9.1.1 Setting the parameters of the Chemostat-Based Biofilm Fermenter (CBBF)

1. Biofilm assay to check the best culture media for *E. faecalis* to run the chemostat

Biofilms were developed in 96-well plates. The growth of the biofilm was initiated by inoculating 10 μl of *E. faecalis* in BHI overnight culture into 300 μl of different types of media (TSB (tryptic soy broth), TSB+YE (yeast extract), BHI (brain heart infusion) all either without glucose, with 0.25% glucose, or 2% glucose) in the individual wells of a 96-well microtiter plate using three different BHI overnight cultures. Wells without cells were used as blank controls. The microtiter plates were then incubated at 37°C in air without CO₂ overnight without agitation. After the incubation, the planktonic cells were carefully removed and the plates were air dried overnight. The plates were then stained with 0.01% (wt/vol) safranin for 10 min, rinsed with sterile distilled water, and air-dried. Biofilms were quantified by measuring the absorbance of stained biofilms at 490 nm with a microplate reader.

**Results (Fig 9.1):** BHI + 2% glucose had the second highest reading after TSB+YE+ 2% glucose (the difference was minor) but with more uniform distribution for all the wells.

![Biofilm assay for *E. faecalis*](image)

**Figure 9.1**: Biofilm assay to check the best culture media for *E. faecalis.*
2. Biofilm experiment to test the appropriate dilution for BHI
(The same methodology as explained in the previous section) using three independent 
*E. faecalis* overnight cultures and three different concentrations of BHI+ 2% glucose 
(undiluted, ½ BHI, ¼ BHI).

**Results** *(Fig. 9.2)*: undiluted and ½ dilution BHI+2% glucose were very close in readings 
to each other and significantly different than ¼ dilution, but the biofilm surface of ½ BHI 
was more uniform by naked eye inspection, so the decision was to use ½ BHI with 2% 
glucose for the above mentioned reason and to decrease the costs of the materials.

![Biofilm assay for different dilutions of BHI](image)

**Figure 9.2**: Biofilm assay for *E. faecalis* growth using different BHI concentrations (¼, 
½, and 1) enriched with 2% glucose.
3. Check the efficiency of different MOPS and phosphate citrate buffer concentrations on the pH of *E. faecalis* overnight culture

**Results (Table 9.1):** Both buffers with different concentrations were not able to maintain the pH around 7. Accordingly we decided to use 40mM phosphate-citrate [439], as this small concentration was more effective in buffering the overnight culture when compared to all the used MOPS concentrations and it did not increase the OD of the overnight culture too much.

- To maintain a neutral pH while running the CBBF a base (1N KOH) was used to control the pH.

**Table 9.1:** The efficiency of different buffers to control the pH of *E. faecalis* o/n culture.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>OD(_{600nm})</th>
</tr>
</thead>
<tbody>
<tr>
<td>No buffer</td>
<td>4.19</td>
<td>1.566</td>
</tr>
<tr>
<td>MOPS 0.1 M</td>
<td>4.27</td>
<td>0.796</td>
</tr>
<tr>
<td>MOPS 0.15 M</td>
<td>4.33</td>
<td>0.725</td>
</tr>
<tr>
<td>MOPS 0.2 M</td>
<td>4.36</td>
<td>0.596</td>
</tr>
<tr>
<td>MOPS 0.25 M</td>
<td>4.25</td>
<td>0.736</td>
</tr>
<tr>
<td>MOPS 0.3 M</td>
<td>4.25</td>
<td>0.744</td>
</tr>
<tr>
<td>Ph-Cit 40 mM</td>
<td>4.55</td>
<td>1.852</td>
</tr>
<tr>
<td>Ph-Cit 60 mM</td>
<td>4.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Ph-Cit 80 mM</td>
<td>4.8</td>
<td>2.696</td>
</tr>
<tr>
<td>Ph-Cit 0.1 M</td>
<td>4.91</td>
<td>1.968</td>
</tr>
</tbody>
</table>
4. Experiments to check if the dental materials inhibit the growth of *E. faecalis*

A. Biofilm assay to check the effect of different types of dental materials on the number of *E. faecalis* biofilm cells

The bottoms of the 96-well plate were covered with one of six different dental materials; no material, composite, total-etch (Scotch-Bond) adhesive (SB), self-etch Easy Bond adhesive (EB), epoxy resin (AH plus) sealer, or Bioceramic sealer (BC). The dental materials were applied to the bottom of the wells and left to dry overnight in the incubator at 37°C. The growth of the biofilm was initiated by inoculating 10 μl of *E. faecalis* in BHI overnight culture into 300 μl of 1/2 BHI with 2% glucose. The microtiter plates were then incubated at 37°C overnight. After the incubation, the planktonic cells were carefully removed and the wells were washed with 200 μl of PBS. The wells were filled with 300 μl of sterile PBS and the bottoms were scratched using sterile pipet tip to disintegrate and collect the biofilm cells. After that, the PBS with the harvested cells was used for serial dilution, plating, and counting the CFU/ml of different types of dental materials.

**Results (Fig. 9.3):** All dental materials showed a decrease in biofilm cells number compared to no dental material.

![Biofilm cells](image)

**Figure 9.3:** Biofilm assay to check the effect of different types of sealers on the number of *E. faecalis* biofilm cells.
B. Inhibition zone experiment

To check the inhibition zone 3 discs of each dental material were prepared and added over BHI mixed with *E. faecalis* top agar plates (top agar: prepared by adding 50% less agar to BHI than the needed to make agar plates). The mixture was prepared by adding 500 µl of *E. faecalis* culture (optical density at 600 nm [OD600] of 1.0) to 3 ml of BHI top agar and poured on BHI plates, and then the plates were incubated overnight at 37°C and assessed for zones of inhibition around each disc.

**Results (Fig. 9.4, Table 9.2):** The only zone of inhibition was noticed for AH plus

![A B C D](image)

**Figure 9.4:** The ability of different sealers to inhibit the growth of *E. faecalis*
A: disc of AH plus with zone of inhibition, B: discs of SB, C: disc of EB, and D: disc of AH (on the right), and disc of BC originally the same size.

**Table 9.2:** The ability of different sealers to inhibit the growth of *E. faecalis*.

<table>
<thead>
<tr>
<th>Material</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoxy Resin Sealer (AH Plus)</td>
<td>Yes / 1mm of inhibition</td>
</tr>
<tr>
<td>Bioceramic Sealer (BC)</td>
<td>No/ the materials expands after setting</td>
</tr>
<tr>
<td>Composite (RC)</td>
<td>No</td>
</tr>
<tr>
<td>Total-etch (Scotch-Bond) Adhesive (SB)</td>
<td>No</td>
</tr>
<tr>
<td>Self-etch Easy Bond (EB)</td>
<td>No</td>
</tr>
</tbody>
</table>
5. An experiment to check the efficiency of the technique used in the previous experiment (experiment #4: Biofilm assay to check the effect of different types of sealers on the number of *E. faecalis* Biofilm cells) to collect the biofilm cells

The biofilms were grown in a 96- well plate on top of six different dental materials; no material, composite, ScotchBond (SB), EasyBond (EB), epoxy resin sealer (AH Plus), or Bioceramic sealer (Endosequence) as described in experiment #4. The bottoms of the wells were dried after the collection of the PBS and the disintegrated biofilm cells, the plates were then stained with 0.01% (wt/vol) safranin for 10 min, rinsed with sterile distilled water, and air-dried. Biofilm surfaces were compared to the control surfaces where no cells were added to check if all the cells were harvested by measuring the absorbance of stained biofilms at 490 nm with a microplate reader.

**Results:** The dental materials absorbed the stain and accordingly the quantification of the remaining biofilm cells was not possible, but according to the staining there was no significant difference in color between the harvested surfaces and the control surfaces with no cells.
6. Determining the flow rate of CBBF (Fig 9.5)

An overnight culture of *E. faecalis* ATCC 29212 in BHI was used to inoculate the CBBF, 1% of the working volume of the chemostat vessel is used; therefore 2 ml of the overnight culture was introduced into the vessel that contains 200 ml of 1/2 BHI + 2% glucose+ 40mM phosphate-citrate buffer, then run for 5 h, to reach an OD of almost 1 before pumping fresh media into the vessel at a dilution rate of 0.5/h (flow rate (F)= 100 ml/h). The dilution rate (D) is defined as the rate of flow of medium over the volume of culture in the chemostat (at steady state the specific growth rate (μ) of the microorganism is equal to the dilution rate). Prior to the insertion of the samples, the CBBF is run for 20 h allowing for the bacterial growth to reach a steady-state; 10 vessel volumes of media had been used before samples inoculation [414].

Flow rate (F) = Dilution (D) X volume (V).
F = 0.5X200= 100 ml/h
Time= V/F=200/100= 2 h for one generation
To reach a steady state the CBBF should be run for 10 generations (30 h) before adding the samples.
The inflow pump rate is (1.67 ml/m), accordingly the total media that we need is 9.3 L to run the chemostat (20 h to reach steady state + 72 h to get mature biofilm).

![Schematic diagram of CBBF](image-url)
9.1.2 An attachment to hold the handpiece in a vertical position

Finding a special attachment to hold the handpiece in a vertical position on the tooth structure to ensure that the Bur (ParaPost®) cuts completely straight in the canals.

Figure 9.6: A special attachment to hold the handpiece in a vertical position.

9.1.3 Preparation of Simulated Human Saliva Esterases (SHSE)

SHSE have been shown to degrade resin composites and adhesives. It was prepared by mixing PCE (pseudocholine esterase) and CE (cholesterol esterase) solutions in levels corresponding to activities present in human saliva (16 units/ml for CE activity and 0.01 units/ml for PCE activity). First, 3 ml PBS was mixed per 1 mg of CE powder to make source CE, then 0.125 ml of source CE was added to 3.5 ml PBS to make Overnight CE. 1.5 ml Overnight CE was then added to 27 ml PBS to make a 32 Unit/ml CE solution that was tested by using para-nitrophenyl butyrate (pNPB) substrate. One unit of CE activity is defined as the release of 1 nmol of p-nitrophenyl per m at 401 nm. PCE has been shown to be stable at room temperature over a 10-day period. The protocol can be used to make a large enough quantity of PCE that lasts for the first 10 days of incubation. 1.75 ml PBS was mixed with 5 mg PCE powder to make source PCE. 53 μl of source PCE was then added to 16.165 ml PBS to make a 0.02 Unit/ml PCE solution which was tested by using butyrylthiocholine iodide (BTC) and 5, 5- dithio-bis (2-nitrobenzoic acid) (DTNB) as enzyme substrate (BTC/DTNB assay). One unit of PCE activity is defined as the release of 1 mmol of 5-thio-2-nitrobenzoic acid per
m at 405nm. Lastly, 32 Unit/ml CE solution and 0.02 Unit/ml PCE solution were then mixed in a 1:1 ratio to make SHSE. All solutions were sterile filtered using a 0.22 μm filter. Considering the stability issue of CE, the SHSE solution should be made fresh for replenishing or replacement in the biodegradation experiments.

9.1.4 Sample definition and explanation for the bacterial proliferation study

A sample in this study means the coronal 3 mm of an anterior root. From each root one sample only was obtained. Three experimental samples were assigned for each group, and three regions of interest (ROI) from each sample were examined under the microscope. The figures 3.1- a, b, and c represent one region of interest for one of the samples.

Regarding the use of tapered gutta percha (GP), the canal was wide enough to use the GP points one in the correct direction, and one reversed (the wider side is down), and the rest of the spaces were filled with small accessory points with tiny taper.
9.1.5 Repeating the one-month pre-incubation experiment for bioceramic sealer

The one month pre-incubated BC groups were not amenable for testing under the CLSM, therefore the experiment was repeated. The second trial showed the same results; the interface was not clear under the microscope and this might be due to fluid absorption and expansion of this sealer. The results were supported by:

1. SEM and Confocal images that showed expansion of the BC sealer, while there was shrinkage and gaps for the SE and TE methacrylate adhesive groups (Fig 9.7, and 9.8)
2. Discs of different material that showed expansion of Bioceramic sealer after setting (Fig. 9.4)

Figure 9.7: SEM micrographs for of interface between root canal dentin (up), and root canal filling (down) of SE (self-etch) and TE (total etch adhesive) adhesive show a gap between the filling and the root-dentin, and a gap-free interface for ER (epoxy resin sealer) and BC (Bioceramic sealer).
Figure 9.8: Confocal images of interface (yellow line) between root canal dentin (right), and root canal filling (left) of A: BC sealer where the interface is not clear, B: of Self-etch adhesive there is a gap between the filling and the root-dentin, and C a clear gap- free interface for epoxy resin sealer.

9.2 Supplementary data for the collagenolytic activity from *E. faecalis*

9.2.1 Growth curve of *E. faecalis* in CDM

To check the effect of *E. faecalis* on human dentinal collagen matrix degradation, the bacteria was incubated in chemically defined media (CDM), to reduce the amount of amino acid content in the media and consequently reducing the background noise for the hydroxyproline analysis.

The first step was to assess the ability of *E. faecalis* to grow in CDM.

**Results (Fig. 9.9):** *E. faecalis* grew well in CDM.

![E. faecalis growth curve](image)

**Figure 9.9:** Growth curve of *E. faecalis* in CDM and BHI.
9.2.2 Finding the proper technique to demineralize the dentinal samples and processing them for SEM imaging

1. Three demineralization techniques were checked (10% Phosphoric acid for 18 h or 7 days, or 7 days 0.1 mol acetic acid), the three were enough to do complete demineralization of the dentin samples according to the EDS results. But again the images did not show a clear collagen network (Fig 9.10).

2. The second step is to check the demineralization using 35% Phosphoric acid for 15 second (to mimic the clinical situation, hoping to protect the collagen fibers from denaturation by excessive exposure to acid), the results again were not as expected (Fig. 9.11).

3. Working on finding a different technique for samples preparation and dehydration. The technique that recommends using HMDS (hexamethyldisilazane) instead of oven drying was followed. Dentin sections were demineralized with 10% Phosphoric acid. For dehydration; the dental fragments were immersed in an increasing concentration of ethanol (25% to 100%), followed by subsequent immersion in 100% acetone, 50% acetone plus 50% HMDS, 100% HMDS and placed in a desiccator for 24 h to obtain complete drying, then gold coated. The results were positive and the procedure was adopted to process the samples incubated with bacteria (Fig. 9.12, and 6.3).
Figure 9.10: SEM of dentin demineralization using three different techniques (10% Phosphoric acid for 18 h or 7 days, or 7 days 0.1 mol acetic acid) at different magnifications (1, 5, or 10 K).

Figure 9.11: SEM of dentin demineralization using 35% Phosphoric acid for 15 sec (10K).

Figure 9.12: SEM of dentin demineralization using 10% Phosphoric acid at 10, and 20K.
Reference


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