Investigation of antimicrobial properties of root canal medicaments on multi-species bacterial-fungal biofilms

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List of Abbreviations

AM                          Alpha-mangostin
ANOVA                       Analysis of variance
AP                           Apical periodontitis
ATCC                        American Type Culture Collection
BHI                         Brain heart infusion
Ca(OH)$_2$                  Calcium hydroxide
CFU                         Colony forming units
CHX                         Chlorhexidine
CLSI                        Clinical Laboratory Standards Institute
EDTA                        Ethylenediaminetetraacetic acid
EPS                         Extracellular Polymeric Substance
EUCAST                      European Committee on Antimicrobial Susceptibility Testing
FDA                         Food and Drug Administration
HICA                        2-hydroxyisocaproic acid
HicDH                       Hydroxyisocaproic acid dehydrogenase
KICA                        2-ketoisocaproic acid
LTA                         Lipoteichoic acid
LPS                         Lipopolysaccharide
MBC                         Minimum bactericidal concentration
MIC                         Minimum Inhibitory Concentration
MRSA                        Methicillin-resistant *Staphylococcus aureus*
NaOCl                       Sodium hypochlorite
OD                           Optical density
PBS                         Phosphate-buffered saline
PCR                         Polymerase chain reaction
PMA                         Propidium monoazide
PMA- qPCR                   Quantitative polymerase chain reaction with propidium monoazide
SAB                         Sabouraud dextrose agar
V/V                         Volume/Volume
W/V                         Weight/Volume
XTT                         2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2Htetrazolium hydroxide
THE UNIVERSITY OF MANCHESTER

ABSTRACT

Investigation of antimicrobial properties of root canal medicaments on multi-species bacterial-fungal biofilms

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Warat Leelapornpisid December 2018

Multi-species bacterial-fungal biofilms are common in the spectrum of infectious diseases including root canal infections. In endodontic treatment, biofilm removal is accomplished by a chemo-mechanical process. Using existing medicaments and techniques, effective disinfection of dentine during root canal treatment remains a major challenge. There is an argument for development of novel antimicrobial agents with a different mode of action, but the potential tissue toxicity and allergic reactions of any alternative medicament must be considered.

The work described in this thesis aims to evaluate the effectiveness of two novel antimicrobial agents, HICA and alpha-mangostin, against multi-species bacterial-fungal biofilms. Another objective is to study the potential of these agents as a root canal medicament. The planktonic susceptibility testing of HICA and alpha-mangostin against six bacterial strains and two Candida albicans was performed to decide the concentration of each agent to be used in biofilm experiments. The metabolic activity of single and multi-species biofilms after exposure to antimicrobial agents was analysed by a metabolic (XTT) assay. The alterations in the biofilm structure were then determined by bright field microscopy and BacLight™ LIVE/DEAD fluorescence microscopy. Time-kill assays were used to determine the antimicrobial activity of HICA and alpha-mangostin against the planktonic root canal microbes compared to commercial root canal medicaments. The ex vivo multi-species biofilms root canal model was developed to mimic the polymicrobial environment of root canal infections. The anti-biofilm activity of HICA and alpha-mangostin was evaluated against established root canal biofilms. Culture techniques were used to quantify biofilm biomass and viability whereas a qPCR method was used to investigate the viability and composition within the biofilms.

Both HICA and alpha-mangostin demonstrated better efficacy against bacterial, fungal, and multi-species biofilms. These agents exerted superior anti-biofilm activity in infected human dental root canals compared to the commonly used inter-appointment intra-canal medicament (Ca(OH)₂) as they reduced biofilm biomass and viability within biofilms effectively. This study showed that HICA and alpha-mangosin have the potential to be used as inter-appointment medications in the treatment of root canal infections, especially for persistent infections or retreatment cases.
Declaration

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Research Contributions

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Chapter 1

General Introduction
1.1 General overview

Endodontic infections are infections of the root canal system, and microorganisms play essential role in the progression and perpetuation of pulpal and periapical pathologies (Hargreaves, 2010; Nair, 2006; Torabinejad and Shabahang, 2009). Caries, trauma or iatrogenic procedures, induced inadvertently by a physician or surgeon or by medical treatment or diagnostic procedures, are the main underlying conditions for endodontic infections leading to pulpitis and pulp necrosis (Figdor and Sundqvist, 2007). Oral microbes gain access into the root canal system and form sessile microbial communities called biofilms in the root canals (Siqueira et al., 2002a; Ricucci and Siqueira, 2010; Ricucci et al., 2009). Infections can spread from the root canals to the periodontal ligament and bone beyond the root apex, ultimately resulting in apical periodontitis (Siqueira, 2003b). Acute apical abscess is the most common example of extraradicular infection; microbial biofilms are formed on the cementum adjacent to the root apex of endodontically infected teeth (Ricucci and Siqueira, 2008). The necrotic tissues in the infected root canal systems are the environment for microbial colonisation and propagation. In addition, this environment is protected from host defence mechanisms (Siqueira et al., 2002b).

The aim of endodontic treatment is to eradicate and kill the infection from the root canal system and allow the immune system to clear the extraradicular infection (Siqueira, 2001; Haapasalo et al., 2005; Ng et al., 2008). For this, a combination of irrigation with a disinfecting agent and mechanical debridement using files are used (Figini et al., 2008). Typically, this requires multiple visits and between appointments the root canal is filled with an antimicrobial medicament and sealed tightly with a temporary filling in order to prevent microorganisms from reinfecting the root canal system (Sathorn et al., 2009; Su et al., 2011; Özcan et al., 2011; Poggio et al., 2011). Once asymptomatic, the root canal is tightly filled and the crown restored.

The use of disinfectants in root canal irrigation and intra-canal medicaments is necessary as some microorganisms can persist in the complex root canal system despite chemo-mechanical treatment (Ørstavik, 2003). The standard irrigant used in endodontics is sodium hypochlorite; it eradicates the microorganisms and dissolves necrotic and vital organic tissue from the root canal systems (Zehnder et al., 2002). Concentrated sodium hypochlorite is toxic and has potential to cause severe clinical
problems such as swelling and haematoma if extruded into vital tissues (Gernhardt et al., 2004). Chlorhexidine digluconate (CHX) is also used as a root canal irrigant, and it has broad-spectrum antibacterial activities against microorganisms found in infected root canals (Siqueira et al., 2007). In addition, CHX has substantivity and binds to dentine (Rosenthal et al., 2004). However, CHX does not dissolve pulp tissue; therefore, it cannot replace sodium hypochlorite as an irrigant (Haapasalo et al., 2010).

The microorganisms persisting in the root canal may proliferate between appointments (Siqueira, 2003a). To manage the root canal infection, an intra-canal medicament should be placed. The ideal properties of medication are to inhibit proliferation and further eliminate remaining microorganisms; moreover, it should minimise ingress of infection via a leaking restoration (Hargreaves, 2010). Calcium hydroxide is the first choice as a root canal medicament; it has anti-bacterial effects due to its high pH (12.5-12.8) (Farhad and Mohammadi, 2005). Moreover, bacterial products such as lipopolysaccharide are denatured and detoxified by calcium hydroxide (Tanomaru et al., 2003). As well as its antimicrobial effects, calcium hydroxide also induces hard tissue formation (Faraco and Holland, 2001). There have also been many attempts to use natural products as root canal irrigants and medicaments. Such products should eliminate pathogenic microorganisms in the root canal systems; moreover, they should be safe in contact with human tissues. D,L-2-hydroxyisocaproic acid (HICA) and 1,3,6-trihydroxy-7-methoxy-2,8-bis (3-methylbut-2-enyl) xanthen-9-one (alpha-mangostin) are natural products which possess both properties. The potential antibacterial properties of natural products may render these materials suitable as alternative root canal irrigants and medicaments.

HICA could be a novel and safe topical antimicrobial agent because of its antimicrobial properties; it has a broad-spectrum of antimicrobial activity against bacterial and against fungal species (Hietala et al., 1979; Sakko et al., 2014; Sakko et al., 2012). HICA is a by-product of the leucine-acetyl-CoA pathway in the leucine metabolism pathway and thus produced and metabolised by human cells (Hoffer et al., 1993; Dallmann et al., 2012). It is also a protein fermentation product of Lactobacillus plantarum, and naturally found in various food products (Additives and
Food, 2012). The enzyme needed for the elimination of HICA is hydroxyisocaproic acid dehydrogenase (HicDH). Few microbes produce this enzyme and HICA is toxic to them. *Lactobacillus* species that can produce HicDH are resistant to HICA (Hummel et al., 1985; Yamazaki and Maeda, 1986; Lerch et al., 1989; Kallwass, 1992; Broadbent et al., 2004; Smit et al., 2004; Smit et al., 2005; Chambellon et al., 2009). HICA has been shown to be fungicidal against planktonic *Candida albicans* and inhibit the formation of *C. albicans* biofilms (Nieminen et al., 2014b). However, the anti-biofilm activities of HICA on bacterial or multi-species biofilms have not been studied so far.

Alpha-mangostin is the derivative from the extracts of the peel of the mangosteen fruit (*Garcinia mangostana* Linn.); it has been shown to possess antimicrobial activity against a wide range of microorganisms (Pedraza-Chaverri et al., 2008; Suksamrarn et al., 2006). The planktonic MIC of alpha-mangostin for various bacteria and fungi ranges between 12,500-50,000 mg/L and 1,000-5,000 mg/L, respectively (Chin et al., 2008). The anti-biofilm activities of alpha-mangostin on bacterial or fungal biofilms have not yet been explored.

The present series of studies focuses on the antimicrobial spectrum of HICA and alpha-mangostin. Antimicrobial activity of both agents was observed for a spectrum of endodontopathogenic microorganisms including bacteria and fungi. The impact of both agents on biofilm structures was evaluated. A particular focus of this study was the potential of HICA and alpha-mangostin for root canal medication. The anti-biofilm activity of both agents against multi-species bacterial-fungal biofilms was observed *ex vivo* in the infected root canals of extracted human teeth for seven days. These test conditions mimic the clinical conditions in an infected root canal system. Moreover, a molecular technique was used to quantify as well as analyse the composition in the root canal biofilm models.

The hypothesis of this study was that HICA and alpha-mangostin are potential antimicrobial agents for the treatment of topical infections, and in addition, as root canal medicaments. The purpose of this study was to evaluate the antimicrobial spectrum and the suitability of HICA and alpha-mangostin for the management of infections in dental root canals in an *in vitro* biofilm and an *ex vivo* root canal model.
1.2 Microbiology of endodontic infections

Many types of microorganisms are involved in endodontic infections including bacteria, fungi, viruses and parasites (Saboia-Dantas et al., 2007; Siqueira and Sen, 2004b; Vianna et al., 2006). However, the major contributors to the pathogenesis of apical periodontitis are bacteria, especially anaerobic species (Figdor and Sundqvist, 2007; Siqueira Jr and Rôças, 2008). Candida spp. is the most common fungal pathogens detected in endodontic infections (Baumgartner et al., 2000; Siqueira et al., 2002c; Siqueira and Sen, 2004a). Interplay between bacteria and fungi are found in endodontic infections particularly in therapy-resistant cases (Shen et al., 2005; Siqueira Jr and Rôças, 2008). Biofilms, organised microbial communities, develop and invade the root canal system in the advanced stages of the infectious process (Siqueira et al., 2002a; Love, 2004; Ricucci and Siqueira, 2010; Estrela et al., 2014). In the extraradicular infections, biofilms can form and become attached to the apical root cementum by growing through the apical foramen (Siqueira, 2003b; Ferreira et al., 2004; Wang et al., 2012). Endodontic infections can be classified according to anatomical location of the biofilms: intraradicular, extraradicular, and periapical (Narayanan and Vaishnavi, 2010). They can also be classified as primary, secondary or persistent according to the time at which microorganisms establish themselves in the root canal systems. Specific microbes dominate each classification (Table 1.1) (Siqueira Jr and Rôças, 2008).

Molecular methods such as pyrosequencing have allowed identification of unculturable microorganisms involved with root canal infections (Munson et al., 2002; Sakamoto et al., 2006). These studies have shown that endodontic infections are more complex than previously reported using culture-based methods (Özok et al., 2012). The apical part of the root canal system is more diverse and harbours a more complex microbiome than their coronal part; a distinct ecological niche in the apical region emphasizes the difficulty of eradication of infection (Özok et al., 2012).
**Table 1.1** Genera of candidate pathogens commonly associated with different types of endodontic infections (Siqueira Jr and Rôças, 2008).

<table>
<thead>
<tr>
<th>Chronic apical periodontitis</th>
<th>Acute apical abscess</th>
<th>Secondary/persistent intraradicular infection</th>
<th>Extraradicular infection</th>
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<tr>
<td><strong>Gram-negative</strong></td>
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<tr>
<td>Porphyrmonas</td>
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<td>Porphyrmonas</td>
<td>Porphyrmonas</td>
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<td>Tannerella</td>
<td>Fusobacterium</td>
<td>Tannerella</td>
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<td>Prevotella</td>
<td>Tannerella</td>
<td>Prevotella</td>
<td>Treponema</td>
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<td>Dialister</td>
<td>Prevotella</td>
<td>Dialisler</td>
<td>Treponema</td>
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<td>Fusobacterium</td>
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<td>Campylobacter</td>
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<tr>
<td>Treponema</td>
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<tr>
<td><strong>Gram-positive</strong></td>
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<tr>
<td>Filifactor</td>
<td>Parvimonas</td>
<td>Enterococcus</td>
<td>Actinomyces</td>
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<td>Eubacterium</td>
<td>Peptostreptococcus</td>
<td>Actinomyces</td>
<td>Propionibacterium</td>
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<td>Actinomyces</td>
<td>Streptococcus</td>
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<td>Parvimonas</td>
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<td>Pseudomonas</td>
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<td>Propionibacterium</td>
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<td><strong>Fungi</strong></td>
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<td>Candida</td>
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Intraradicular infections can be sub-categorized into primary and secondary/persistent infections. Primary infections are characterised by mixed microorganisms and involve tens of species per canal (Munson et al., 2002; Siqueira and Rôças, 2005; Siqueira et al., 2004). Up to 10^8 bacterial cells per root canal have been reported (Sakamoto et al., 2007; Vianna et al., 2006). The predominant bacterial species in primary infections are obligate anaerobic bacteria (Narayanan and Vaishnavi, 2010). The microorganisms found in endodontically-treated teeth may be survivors from the effects of intracanal disinfection procedures and were present in the canal at the root canal-filling stage, termed “persistent intraradicular infections”. The microorganisms infecting the canal after filling as a result of coronal leakage are called “secondary intraradicular infections”. Microorganisms in root canal-treated teeth with persistent apical periodontitis exhibit a decreased diversity when compared with primary infections (Pinheiro et al., 2003; Siqueira and Rôças, 2004). The predominant bacterial species in secondary/persistent infections are
facultative anaerobes, especially *Enterococcus faecalis* (Narayanan and Vaishnavi, 2010). Sometimes, fungi are found in small amounts (Siqueira and Sen, 2004a): *Candida albicans* is the most commonly detected fungal species in endodontically-treated teeth (Pinheiro et al., 2003). The root apex acts as an effective barrier to spread of infection into the extraradicular space. Nonetheless, in some clinical situations, microorganisms overcome this defence barrier and establish an extraradicular infection, which can be dependent on, or independent of, the intraradicular infection (Siqueira, 2003b).

### 1.3 Biofilms

A biofilm can be defined as a structured consortium of aggregated microbial cells that typically adhere to various surface structures and are enmeshed in a self-produced matrix of extracellular polymeric substance (Siqueira Jr and Rôças, 2008). Biofilms can also form in a cavity as non-adherent microcolonies, and form free floating biofilm balls (Loussert et al., 2010; Kernien et al., 2017). The significant structure of mature biofilms is micro-colonies of microorganisms embedded in a matrix which contains open water channels to accommodate the flow of water and nutrients dissolved into it (Hall-Stoodley et al., 2004; Stoodley et al., 2002). There are many advantages for microorganisms forming a biofilm. Microbes within biofilms are more virulent and have inherently increased resistance to antimicrobial agents when compared with the same microbes grown under planktonic conditions (Hall-Stoodley et al., 2004; Lynch and Robertson, 2008; Parsek and Singh, 2003).

To form biofilms, planktonic free-floating microbes in a liquid environment are required. The important factor for the initial attachment of organisms is the excretion of adhesive substances such as polysaccharides and proteins (Czaczyk and Myszka, 2007). Extracellular DNA (eDNA) is also an adhesive substance which plays an active role in promote or modulate biofilm development (Izano et al., 2009). The adhesive substances also hold the biofilm together, the adhesion of microorganisms to a surface can trigger an alter expression of a large number of genes; as a consequence, the phenotypes of microorganisms are changed (Ramage et al., 2011). However, the local environmental conditions within the biofilm may also cause the alteration of gene expressions and protein synthesis (Svensäter and Bergenholtz, 2004; Stewart and Franklin, 2008; Verstraeten et al., 2008). The dense
aggregates of bacteria in biofilms result from quorum sensing: a bacterial cell-to-cell communication mechanism for controlling cellular functions. The role of quorum sensing signaling is to regulate several microbial properties, including virulence and the ability to form biofilms; moreover, this signaling can incorporate extracellular DNA and cope with environmental stress as well (Cvitkovitch et al., 2003; Dobretsov et al., 2009; Rutherford and Bassler, 2012; O'Loughlin et al., 2013).

1.3.1 Biofilm formation

Biofilm formation usually begins with adhesion of planktonic microbes to a surface. Then, colonisation, co-adhesion, growth, and maturation occur respectively (Gergova et al., 2015; Hall-Stoodley and Stoodley, 2005). The formation of a biofilm is divided into three stages (Figure 1.1).

![Figure 1.1 Stages of biofilms formation (Svensäter and Bergenholtz, 2004).](image)

In the first stage, planktonic microbes must adhere to a biomaterial surface (O'Toole et al., 2000). A film is formed by adsorption of macromolecules in the planktonic phase to the surface (Allison, 2000). The film has the selectively adhesion properties decided by the early colonisers (such as Porphyromonas gingivalis) which other microorganisms can attach to, and thereby influences the microbial composition of the biofilm. The initial attachment is mediated by both nonspecific factors including cell surface hydrophobicity and electrostatic forces, as well as by specific adhesins on the microbe surface that recognise ligands in the biofilms, such as serum proteins (fibrinogen and fibronectin) (Li et al., 2003; Nett and Andes, 2006; Ramage et al., 2005). Moreover, interspecies attachment also occurs. Fungal cells can directly attach to one another or to bacterial organisms that have already
colonised the biomaterial (El-Azizi et al., 2004).

In the second stage, adhesion and co-adhesion of microorganisms occur. For bacterial biofilms, the attachment may be strengthened through polymer production and unfolding of cell surface structures. The specific early colonisers play important roles which results in subsequent co-adhesion of other organisms (Kolenbrander et al., 2002; Castonguay et al., 2006). For fungal biofilms, growth ensues and micro-colonies are formed. Fungal cell begins to multiply by budding, filamentous scaffolding is produced and the initial deposition of extracellular matrix material occurs and subsequent biofilm development follows (Martinez and Fries, 2010; Du et al., 2012; Harding et al., 2009; Nett et al., 2007). Filamentous growth strengthens the entire biofilm structure and provides protection and adhesion sites for the budding yeast cells (Ramage et al., 2005; Richard et al., 2005; Du et al., 2012; Inglis et al., 2013). Micro-colonies are later conjoined by hyphal extensions leading towards a confluent monolayer (Ramage et al., 2001a). Yeast cells make up the basal layer, while filamentous cells compose the structural framework (Baillie and Douglas, 2000; Ramage et al., 2001b; Banerjee et al., 2013; Uppuluri et al., 2010; Araújo et al., 2017).

In the third stage, multiplication and metabolism of attached microorganisms occurs, and the structurally organised mixed microbial community is formed. For bacterial biofilms, the late colonisers (such as Actinobacillus actinomycetemcomitans) attach to the early colonisers by co-aggregation or co-adhesion. Moreover, Fusobacterium nucleatum, a gram-negative anaerobe, can act as a bridge between the early and the late colonisers (Rickard et al., 2003; Periasamy and Kolenbrander, 2009). For fungal biofilms, the quantity of the extracellular material which consists of proteins, chitins, DNA, and α-1,3 glucan carbohydrates increase in a time dependent manner until the microbial communities are entirely enclosed to form a mature biofilm (Al-Fattani and Douglas, 2006; Chandra et al., 2001). For multi-species biofilms, the interactions between microbes affect their overall function and physiology as well as host responses and result in enhanced resistance and virulence (Ren et al., 2014; Liu et al., 2016; Røder et al., 2016). Mixed bacterial-fungal biofilms develop through coaggregation (Peleg et al., 2010) and mature mixed biofilms consist of a yeast base with hyphal structures forming a complex network surrounded by bacteria and encased in ECM (Harriott
and Noverr, 2011). The detachment of microorganisms from a biofilm structure is a continuous process in order to spread and colonise other sites. Localised detachment of microorganisms starts after initial adhesion and increases with time as it is related to the number of microorganisms present in the biofilm (Svensäter and Bergenholtz, 2004).

1.3.2 Biofilms in endodontic infections

Most cases of apical periodontitis involve biofilms (Mohammadi et al., 2013). Endodontic biofilms can be formed on both root canal structures (intraradicular, extraradicular, periapical microbial biofilms) and biomaterial-centered infections (pre-treated root canals filled with gutta percha) (Narayanan and Vaishnavi, 2010; Al-Ahmad et al., 2014). The endodontic microbiome changes significantly in the progression of infection as the anaerobic environment and limited nutrition within the root canal offer a tough ecological niche for the microorganisms. Moreover, the anatomical complexity of the root canal system shelters the microbes from mechanical instrumentation (Jhajharia et al., 2015). Biofilms are formed and invade into the root canal dentine of an endodontically infected tooth (Ingle et al., 2008; Nair, 2006; Sundqvist and Figdor, 2003). There is a high prevalence of bacterial biofilms in the apical root canals of both untreated and treated teeth with apical periodontitis (Ricucci and Siqueira, 2010). Root canal microbes can invade the extraradicular area via the apical foramen and lead to biofilm formation on the apical cementum (Noiri et al., 2002). Extraradicular and periapical biofilms have been found to be associated with refractory periapical periodontitis (Frank and Barbour, 2006; Ricucci et al., 2016; Noguchi et al., 2005).
Fungal biofilms are also present in endodontic infections which contribute to the aetiology of periapical diseases. The pathogenesis of diseases originating from fungal virulence factors includes adaptability to a variety of environmental conditions, adhesion to a variety of surfaces, the production of hydrolytic enzymes, morphologic transition, biofilm formation, and evasion and immunomodulation of the host defense (Nair, 2006). Fungi can gain access to the root canals through contamination during endodontic treatment and they can be involved in the aetiology of recalcitrant or persistent periradicular lesions (Nair, 2006; Siqueira et al., 2002c; Waltimo et al., 2003). In addition, fungi can colonise dentine which may be an important step in infection of the root canal system. They also invade dentinal tubules and are involved in the establishment of persistent endodontic infections (Siqueira et al., 2002c; Siqueira and Sen, 2004a).
1.3.3 Bacterial-fungal interactions in biofilms

The complex structure of the biofilm allows some degree of interspecies cooperation to develop between microbial populations (Elias and Banin, 2012). There are metabolic interactions between microorganisms in biofilms, including mutualistic and commensal relationships (Freilich et al., 2011; Lee et al., 2014). *C. albicans* is the most common fungus associated with oral infections (Douglas, 2003). The majority of research on bacterial-fungal interactions has used on *C. albicans* as the fungal pathogen (Wargo and Hogan, 2006; Morales and Hogan, 2010; Shirtliff et al., 2009). Many studies have been done on *Candida* and *Pseudomonas* in cystic fibrosis models, showing that signaling occurs between these two species (McAlester et al., 2008; Bandara et al., 2010; Hogan et al., 2004; Morales et al., 2013). Studies on bacterial-fungal oral biofilms, such as *P. gingivalis, Pseudomonas aeruginosa-C. albicans* biofilms, have shown that the microbes communicate by signaling and this has role in the pathogenesis (Kolenbrander et al., 2010; Thein et al., 2006; Shirtliff et al., 2009). There are also physical interactions between *C. albicans* and biofilm-forming pathogenic bacteria, the surface polysaccharides play an important role in the colonisation of bacterial biofilms by *C. albicans* and vice versa (El-Azizi et al., 2004). The bacteria which produced glycocalyx were able to better adhere to *C. albicans* biofilms. By contrast, the fungus could not attach as easily to preformed biofilms of glycocalyx-producing bacteria. Moreover, Klotz et al. studied the mixed microbial aggregates from the interactions between *C. albicans* and bacteria were found (Klotz et al., 2007). The adhesion process to host surfaces needs cell-surface glycoproteins like *C. albicans* agglutinin-like sequences (Als), which are essential in co-adhesion of mixed microbial communities in biofilms and on mucous surfaces. In addition, *E. faecalis* and *C. albicans* have a negative impact on each other; *E. faecalis* inhibited *C. albicans* hyphal morphogenesis. This inhibition was partially dependent on the Fsr quorum-sensing system. GelE and SerE, which are proteases regulated by Fsr, were partially required (Cruz et al., 2013). Mixed bacterial–fungal biofilms are always present in the oral environment, including infected root canal (Ferrari et al., 2005) and root caries (Shen et al., 2005). In persistent endodontic infections, *C. albicans* can be isolated with *Peptostreptococcus micros* in mixed infections from root canal samples (Jabra-Rizk et al., 2001; Lana et al., 2001), suggesting that *Candida* may play a role in therapy-resistant apical periodontitis and root canal infections with pulp necrosis (Siqueira
1.4 *Enterococcus faecalis*

*Enterococci* are gram-positive facultative anaerobic coccoid bacteria. Enterococcal cells are oval-shaped and 0.5 to 1 μm in diameter. Planktonic enterococci can exist as single cells, in pairs or as short chains (Rôças et al., 2004b). *Enterococci* grow at temperatures ranging from 10-45°C, in a wide range of pH (4.6–9.9) (Van den Berghe et al., 2006), they also tolerate and grow in the presence of 40 % (w/v) bile salts. *Enterococci* are able to grow in 6.5 % NaCl; they can survive at 60°C for 30 minutes (Stuart et al., 2006). *Enterococci* are recognised as opportunistic pathogens and are commonly found in the oral cavity, normal intestinal microflora, and in the genital area of both humans and animals (Mohamed and Huang, 2007). Of the enterococcal group, *E. faecalis* is considered to be the most significant species, being not only frequently isolated from sites of infection but also resistant to antibiotics having an antibiotic resistant property (Fisher and Phillips, 2009). *E. faecalis* has been isolated from some oral conditions including carious lesions, chronic periodontitis, and persistent apical periodontitis (Zhu et al., 2010). It has also been detected primarily at sites of persistent endodontic infections (Bonten et al., 2001; Kaufman et al., 2005; Pinheiro et al., 2003; Rôças et al., 2004a) and some primary endodontic infections (Gomes et al., 2008; Kaufman et al., 2005; Sassone et al., 2007).

1.4.1 Virulence mechanisms and the factors affecting distribution of *Enterococcus faecalis* in the root canal

Enterococci can persist in a spectrum of environments and they are often resistant to many antibiotics. Oral *E. faecalis* has been shown to possess a range of virulence factors including lytic enzymes, aggregation substance (AS), pheromones and lipoteichoic acid (Stuart et al., 2006). The transfer of certain virulence traits to related species in root canals has been demonstrated (Sedgley et al., 2008). *E. faecalis* can enter a viable but non-cultivable state (VBNC) (Lleo et al., 2001), which is a bacterial survival mechanism on exposure to adverse environmental conditions, including low nutrient concentrations, high salinity, and extreme pH (Lleo et al., 2005). In a VBNC state, bacteria lose the ability to grow in culture media, but they
maintain viability and pathogenicity. In addition, they are able to resume division when favorable environmental conditions are restored (Heim et al., 2002). Moreover, *E. faecalis* has the ability to penetrate into dentinal tubules and facilitate its long-term survival in the root canal system (Siqueira et al., 1996; Love and Jenkinson, 2002; Chivatxaranukul et al., 2008). Depth of tubule penetration by *E. faecalis* has been observed to be over 200 μm by scanning electron microscopy (Chivatxaranukul et al., 2008). Collagen binding protein plays significant roles in binding of enterococcal cells to the root canal wall (Hubble et al. 2003) Moreover, *E. faecalis* can be found as a mono-infection in medicated root canals (Usha, 2010). It has the ability to acquire, accumulate and share extrachromosomal elements encoding virulence traits (Lindenstrauß et al., 2011; Sun et al., 2012). These mechanisms help *E. faecalis* to colonise, compete with other bacteria, resist host defense mechanisms and produce pathological changes (Stuart et al., 2006; Zoletti et al., 2011; Upadhyaya et al., 2010).

Enterococci have been associated with difficult-to-treat biofilm-mediated infections (Paganelli et al., 2012). Many environmental and genetic factors are associated or have been proposed to be associated with the enterococcal biofilm biogenesis (Mohamed and Huang, 2007). Carbohydrate metabolism regulates enterococcal biofilm production (Pillai et al., 2004; Kristich et al., 2004). Moreover, the enterococcal surface protein is involved in biofilm formation in the presence of a higher glucose concentrations (Tendolkar et al., 2004; Creti et al., 2006). Changes in the environment conditions including osmotic strength can also affect the enterococcal biofilm formation (Mohamed and Huang, 2007; Habimana et al., 2014).

1.5 *Candida albicans*

*Candida albicans* is a commensal pathogen that can be isolated from the gastrointestinal tract, upper respiratory tract, oral and vaginal mucosa (Kim and Sudbery, 2011); it has been detected in both healthy and medically compromised individuals (Siqueira and Sen, 2004a). In the oral cavity, *C. albicans* is mainly found at sites where endogenous oral commensal bacteria are also present (Dongari-Bagtzoglou et al., 2009). These polymicrobial communities readily form on the oral mucosa (Dongari-Bagtzoglou et al., 2009), dental plaque and dentine of carious teeth (de Carvalho et al., 2006), infected root canals (Baumgartner et al., 2000;
Gomes et al., 2010) as well as dental prosthetic structures (Daniluk et al., 2006) and orthodontic appliances (Zharmagambetova et al., 2017).

*C. albicans* causes infection when an opportunity arises (Ghannoum et al., 2010; Rautemaa and Ramage, 2011;). The main factors which keep *Candida* under control in the oral cavity are local defence provided by saliva and oral mucosa as well as the competitive inhibition from the other oral microorganisms (Wilson, 2005). Moreover, good daily oral hygiene and mechanical disruption of oral biofilms play an important role in controlling *Candida* (Grimoud et al., 2005). Oral candidosis should be observed as a sign of poor oral hygiene and presence of biofilms, loss of bacterial competition due to antibiotics/narrow spectrum mouthwashes, or impaired host immune responses (Rautemaa and Ramage, 2011).

1.5.1 Virulence mechanisms and the factors affecting distribution of *Candida albicans* in the root canal

Virulence mechanisms of *C. albicans* associated with host recognition, binding to host cells, host cell proteins or microbial competitors (co-aggregation) more than likely prevents or at least reduces the extent of clearance by the host (Calderone and Fonzi, 2001; Mayer et al., 2013). There are many factors related to virulence of *C. albicans*. Firstly, adherence is an important prerequisite in the pathogenesis of oral candidal infections (Blank et al., 2006; Calderone and Fonzi, 2001; Nobile et al., 2008) and a low pH environment supports the growth and colonisation of *Candida* (Davis et al., 2002; Rustchenko et al., 2002). Moreover, *Candida* can produce an enzymatic necrotic factor which is result in a rupture of the structural barriers of host cells during infection. *C. albicans* can stimulate monocytes to induce TNF-α production (Tada et al., 2002). Mannoproteins (cell surface polysaccharides) play an important role in the interaction between *Candida* and host cell receptors (de Groot et al., 2004). In addition, hydrophobic *C. albicans* cells can bind abundantly and diffusely to host tissues free of macrophages (Hazen et al., 2000). Furthermore, the presence of bacteria is a contributing factor in the colonisation of *Candida* strains in the oral cavity as bacteria have been found to regulate the adhesion of *Candida* (Salerno et al., 2011). Morphogenesis is the transition between unicellular yeast cells and a filamentous growth form; *C. albicans* reversibly converts unicellular yeast cells to either pseudohyphal or hyphal growth in
response to environmental conditions (Calderone and Fonzi, 2001). Enzymes that contribute to invasiveness, including the secreted aspartyl proteinases (SAP) and phospholipases (PL) have been associated with virulence (Calderone and Fonzi, 2001). Finally, phenotypic switching, which is switching between two cell-types, results in a change in colonial morphology, and is important in adaptation of the organism to specific anatomical sites (Calderone and Fonzi, 2001). C. albicans has been reported in root canal infections by culture, molecular methods, and electron microscopy (Nair et al., 1990; Siqueira et al., 2002c; Baumgartner et al., 2000). C. albicans in the form of yeast cells and some hyphal structures were observed along root canal walls of the teeth with necrotic pulps (Sen et al., 1995). The migration of candida cells into dentinal tubules at the root canal wall were observed; however, tubule penetration was usually slight and restricted to just a few tubules (Siqueira et al., 2002c).

Ability of C. albicans to form biofilms is one of its virulence factors. Candidal biofilms are resistant to antifungal agents because of low growth rate, matrix production, which may alter the cell surface and form a physical barrier, or gene expression patterns (Nett et al., 2010; Uppuluri and Ribot, 2017; Bonhomme and d'Enfert, 2013). A number of genes involved in all stages of candidal biofilm formation, such as in adherence, matrix production, quorum sensing and especially morphogenesis (Bonhomme and d'Enfert, 2013). The gene expression is under the influence of environmental conditions and regulatory pathways (Biswas et al., 2007; Diezmann et al., 2012). Moreover, the biofilm matrix plays an important role in antifungal tolerance (Nobile and Johnson, 2015). Therefore, the identification of potential antifungal targets is warranted.

1.6 Disinfection in endodontic procedures

Irrigation of the root canal system using disinfectants plays an essential role in root canal treatment (Gonçalves et al., 2016). When multiple visits are required, the root canal is filled with an antimicrobial medicament in order to kill the persisting microbes (Kennedy and Hussey, 2007).
1.6.1 Root canal irrigants

The aims of root canal irrigants are to reduce intraradicular microorganisms and to neutralise their endotoxins (Zehnder, 2006). Irrigants should dissolve vital or necrotic pulpal tissue; they should also lubricate the canal walls and instruments during cleaning and shaping procedures (Zehnder, 2006). They should remove dentine particles and have a broad antimicrobial spectrum (Schäfer, 2007). In addition, they should have tissue-dissolution capability but also be biocompatible with human tissues (Schäfer, 2007). Many clinical studies have shown that copious irrigation with an antimicrobial solution during mechanical root canal instrumentation results in significant reduction of intraradicular microorganisms (Kennedy and Hussey, 2007; Schäfer, 2007). Several irrigants have been used in root canal systems.

1.6.1.1 Sodium hypochlorite

Sodium hypochlorite (NaOCl) is the irrigant widely used in endodontics (Zehnder, 2006). NaOCl dissociates into Na⁺ (sodium ion) and OCl⁻ (hypochlorite ion) in water. In the clinical situation, the entirely available chlorine is in the form of OCl⁻, as the pH of the solution is normally about 12 (Zehnder, 2006; Frais et al., 2001). NaOCl solutions are used at concentrations varying from 0.5% to 5.25% in endodontic treatment (Haapasalo et al., 2005). NaOCl has effective antibacterial activity with comparably short contact times (Haapasalo et al., 2005; Zehnder, 2006). Several microorganisms found in primary root canal infection displayed high susceptibility to NaOCl (Vianna et al., 2004). Conversely, *E. faecalis* is much more resistant to NaOCl than primary endodontic microorganisms (Abraham et al., 2015; Gomes et al., 2001; Peciuliene et al., 2001). Moreover, both 5% and 0.5% NaOCl solutions can kill resistant *C. albicans* (Waltimo et al., 1999). NaOCl has also been reported to be effective against both bacterial and fungal biofilms. Using single species models no growth was detected after exposure to 0.5% or higher concentrations of NaOCl (Özok et al., 2007; Radcliffe et al., 2004). NaOCl dissolves both vital and necrotic pulp remnants, organic compounds of dentine, and the organic components of the smear layer (Naenni et al., 2004). It also has a greater tissue-dissolving capability than all other commonly used irrigants (Naenni et al., 2004). However, the inorganic components of the smear layer are not removed by
NaOCl (Torabinejad et al., 2003). There are rare reports of allergic reactions to NaOCl (Zehnder, 2006), but it is known that NaOCl has a potential risk of provoking hypersensitivity reactions (Dandakis et al., 2000). Using NaOCl can harm other tissues if accidentally extruded through the apex into periapical tissues or adjacent anatomical structures such as the maxillary sinus (Hülsmann and Hahn, 2000). The complications of accidental injection of NaOCl into periapical tissues are emphysema, oedema and paraesthesia (Hülsmann and Hahn, 2000).

### 1.6.1.2 Chlorhexidine

Chlorhexidine (CHX) digluconate is used at concentrations varying from 0.2% to 2.0% in endodontic treatment (Schäfer, 2007). CHX has a wide range of antimicrobial properties (Haapasalo et al., 2005). An in vitro study showed that CHX liquid at 0.2, 1.0, 2.0% concentrations and 2.0% CHX gel eliminated facultative microorganisms (E. faecalis) and C. albicans in 1 minute or less. In addition, 0.2, 1.0, 2.0% CHX solutions can kill the gram-negative strictly anaerobic microorganisms P. gingivalis, P. endodontalis, and P. intermedia in only 15 seconds (Vianna et al., 2004). The antimicrobial mechanism of CHX is its ability to permeate the cell wall or cell membrane of bacteria and yeast (Haapasalo et al., 2005). Many in vitro studies have reported that CHX has a marked antimicrobial effect on E. faecalis after a short contact time even at comparatively low concentrations (Gomes et al., 2001; Önçäğ et al., 2003; Schäfer and Bossmann, 2001; Waltimo et al., 1999). In addition, CHX was found to be a very effective antifungal agent (Waltimo et al., 1999). Several studies have shown that CHX is effective at killing C. albicans; it also has anti-biofilm activity with low MIC values (Lamfon et al., 2004; Redding et al., 2009). CHX can bind to dentine and enamel because of its cationic properties (White et al., 1999). It also has substantivity and is gradually released over time which results in prolonged antimicrobial activity (Frais et al., 2001; Zehnder, 2006). Unlike NaOCl, CHX does not dissolve pulp tissue, it is unable to remove the smear layer or neutralise lipopolysaccharides (Haapasalo et al., 2005). Sensitivity to CHX is very rare (Naenni et al., 2004), but it has been recorded to cause anaphylactic reactions and anaphylactic shock in some cases (Naenni et al., 2004; Garvey et al., 2001).
1.6.2 Root canal medicaments

The general definition of intra-canal medicaments is “temporary placement of medicaments with good biocompatibility into root canals for the purpose of inhibiting coronal invasion of bacteria from the oral cavity” (Tronstad et al., 2000). After root canal instrumentation and irrigation, it is important to use inter-appointment medication to eliminate the remaining bacteria within the root canal systems. Root canal medicaments and dressings are classified as a medicinal product (MHRA, 2016) and regulated under the Human Medicines Regulations 2012 (SI 2012/1916) by the European Medicines Agency (EMA) or by the US Food and Drug Administration (FDA).

The ideal intra-canal medicaments should have the ability to reduce periapical inflammation and pain, and induce healing. Some medicaments are also claimed to help eliminate or reduce apical exudates, control inflammatory root resorption, as well as prevent contamination between appointments (Stuart et al., 2006). Moreover, the capacity to induce hard tissue formation is required for medicaments used in endodontic treatment of immature teeth; ideally medicaments should induce the apex growth in cases of incompletely developed roots (Ørstavik and Pitt Ford, 1998).

1.6.2.1 Calcium hydroxide

Calcium hydroxide (Ca(OH)$_2$) is a strong alkaline substance with a pH of approximately 12.5. It has been used in several clinical situations because of its antibacterial properties and the ability to induce repair and to stimulate hard-tissue formation (Faraco and Holland, 2001). Ca(OH)$_2$ also denatures and detoxifies bacterial products including lipopolysaccharide (Tanomaru et al., 2003). Hydroxyl ions are released from calcium hydroxide in an aqueous environment and they are related to its antimicrobial properties. Hydroxyl ions are highly oxidising free radicals; they kill bacteria by damaging the cytoplasmic membrane, denaturing proteins, as well as damaging the bacterial DNA (Farhad and Mohammadi, 2005). The alkaline pH from calcium hydroxide plays an important role in hard-tissue formation, it not only inactivates osteoclasts by neutralising acidified inflammatory lesions, but also activates alkaline phosphatases (Estrela et al., 1995). However, calcium hydroxide is not effective in eliminating C. albicans and E. faecalis (Ercan et al., 2006), because
these organisms have buffering capacity against the high pH produced by calcium hydroxide (Stuart et al., 2006).

1.7 Novel and alternative intra-canal irrigants and medicaments

Because of certain limitations associated with standard intra-canal irrigants and medicaments, there have been many attempts to investigate novel or alternative substitutes. The irrigants and medicaments are classified as antiseptic which are biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue. These substances should be able to eliminate pathogenic microorganisms in the root canal systems; moreover, they should be safe when used in human tissues. The two main regulatory organisations are European Medicines Agency (EMA) and Food and Drug Administration (FDA). In the past, these endodontic medicaments were classified as medical devices and required CE marking (French-Mowat & Burnett, 2012), which involves a less robust process than licensing under medicinal product by EMA or FDA. HICA and alpha-mangostin are natural compounds which have been shown to possess the antimicrobial and biocompatible properties, but they have not yet been approved by EMA, FDA. Pre-market approval is the required process of scientific review to ensure the safety and effectiveness of these agents. Evaluation of safety is required for all medicinal products before initial approval. The additional indications and alternative dose regimens must always take into account the demonstrated and anticipated benefits and risks (EMA, 2011). Continued development of novel antimicrobials is required due to emerging antimicrobial resistance due to overuse in humans and animal husbandry (Huttner et. al., 2013). Legislative and professional control is required to protect the old and new antimicrobial agents.

1.7.1 HICA

D,L-2-hydroxyisocaproic acid (HICA) is also known as leucic acid, 2-hydroxy-4-methylvaleric acid or 2-hydroxy-4-methylpentanoic acid. It is a derivative of leucine with a molecular weight of 132.16 g/mol. HICA present in plasma at very low concentrations and can be detected as a result of leucine metabolism. Moreover, HICA has also been found as a fermentation by-product of Lactobacillus plantarum (Hietala et al., 1979). In addition, there is evidence that HICA has anti-inflammatory properties through inhibition of host extracellular matrix degrading proteases in vitro.
(Westermarck et al., 1997). As a consequence, HICA can increase muscle mass and speed up recovery after exercise, so it has been used by professional athletes as a nutritional supplement (1500 mg daily dose) (Mero et al., 2010). Moreover, HICA is used as a growth-promoting nutritional supplement in animal feeds (Hietala et al., 2005). Thus, it has a good biocompatibility and safety profile. Several studies have indicated the broad-spectrum bactericidal activities of HICA at a concentration of 36,000 mg/L against gram-positive and gram-negative bacteria (Table 1.2) (Nieminen et al., 2014a; Sakko et al., 2012; Sakko et al., 2014). HICA not only significantly inhibits planktonic growth of C. albicans, but it also inhibits the biofilm formation (Nieminen et al., 2014b). Moreover, HICA can affect the production of mutagenic acetaldehyde (ACH), which is an important mediator of candidal biofilm formation, by reducing its production under certain conditions in vitro. Thus, HICA has the potential to be considered as an appropriate agent for treatment of topical bacterial-fungal biofilm infections (Nieminen et al., 2014b). The mode of action for HICA is still unknown, more transcriptional and proteomic studies are required to clarify the specific factors. A similar role, the expression of mutagenic acetaldehyde (ACH) catabolism genes, has been suggested for cysteine due to its structural similarities and activities to HICA (Nieminen et al., 2014a; Hennicke et al., 2012; Hennicke et al., 2013). Therefore, cysteine may provide clues to understand the mode of action of HICA (Nieminen et al., 2014a).

Many antimicrobial agents and disinfectants lose their efficacy in the presence of organic matter (Haapasalo et al., 2000; Portenier et al., 2001). The antibacterial activity of HICA is not significantly affected by clinically relevant concentrations of dentine and other inhibitors commonly present in the root canal systems including dentine powder, hydroxyapatite, and bovine serum albumin (Sakko et al., 2016). Moreover, the antibacterial activity of HICA against E. faecalis has been demonstrated in human dental root canals ex vivo (Sakko et al., 2017). However, the anti-biofilm activities of HICA on the multi-species root canal biofilms have not been studied yet.
1.7.2 Alpha-mangostin

Mangosteen (*Garcinia mangostana* Linn.), of the family *Guttiferae*, is a tropical evergreen tree originally from Southeast Asia (Kanchanapoom et al., 1998; Ketsa and Atantee, 1998). Mangosteen has been used in Asian traditional medicine for a century; its fruit is used to treat skin infections, wounds, diarrhea, dysentery, suppuration, leucorrhea, chronic ulcers and gonorrhea (Nguyen and Marquis, 2011). Moreover, the pericarp of mangosteen is used for the treatment of diarrhea, skin infection and chronic wounds (Obolskiy et al., 2009) and its extracts have been shown to possess antimicrobial activity against a wide range of microorganisms (Nguyen and Marquis, 2011). Phytochemical studies have shown that mangosteen pericarp extracts comprise several active components belonging to a group of xanthone derivatives: alpha-, beta-, gamma-mangostin, garcinone, mangostanol and gartinin (Suksamrarn et al., 2002). Alpha-mangostin (1,3,6-Trihy-droxy-7-meth-oxy-2,8-bis-(3-methyl-but-2-en-yl)-9H-xanthen-9-one) has the most potent antimicrobial activity against bacteria, fungi, and viruses (Chomnawang et al., 2005; Obolskiy et al., 2009; Suksamrarn et al., 2003; Pedraza-Chaverri et al., 2008; Suksamrarn et al., 2006). Alpha-mangostin also exerts the other biological activities including antioxidant (Fang et al., 2016; Manimekalai et al., 2015), anti-cancer (Kaomongkolgit, 2013; Li et al., 2014), anti-inflammatory (Liu et al., 2012), and anti-allergy (Ibrahim et al., 2016).

Many studies have shown that alpha-mangostin has broad-spectrum antimicrobial activity (Table 1.2). Therefore, it has been used to treat skin infection, diarrhea, tuberculosis, acne and prevent caries (Kaomongkolgit et al., 2009; Suksamrarn et al., 2003; Torrungruang et al., 2013). It has been shown that there is no cytotoxicity to human gingival fibroblast when exposed to alpha-mangostin at 4,000 mg/L for 8 hours (Kaomongkolgit et al., 2009). Alpha-mangostin also showed biocompatibility with human PDL cells (Kaomongkolgit et al., 2013). In human clinical trials, no local irritation or side effects were observed in patients after 1.5% was applied to skin (Rassameemasmaung et al., 2007). The MIC concentration of alpha-mangostin against different bacteria and fungi is 12,500-50,000 mg/L and 1,000-5,000 mg/L respectively (Chin et al., 2008). A study to explain the mode of action of alpha-mangostin in terms of its antimicrobial properties against methicillin-resistant
S. aureus (MRSA) was carried out by Koh (2013). The results indicated that the cytoplasmic membrane was rapidly disrupted by alpha-mangostin, so the intracellular components were lost. Moreover, biophysical studies using fluorescence probes for membrane potential and permeability, calcein-encapsulated large unilamellar vesicles and scanning electron microscopy illustrated that the cell membrane of MRSA was broken down and its permeability consequently increased. This was the result of the penetration of hydrophobic phenyl ring of alpha-mangostin into the lipid bilayer of the bacterial cell membrane. Thus, this study suggests that there are direct interactions between alpha-mangostin and the bacterial membrane that lead to rapid disruption and bactericidal action in a concentration-dependent manner (Koh et al., 2013). The anti-biofilm activities of alpha-mangostin have not been studied. Moreover, the effectiveness of alpha-mangostin has not been tested in the root canal dentine.
Table 1.2 Antimicrobial activity of sodium hypochlorite (NaOCl), chlorhexidine (CHX), calcium hydroxide (Ca(OH)\(_2\)), HICA, and alpha-mangostin.

<table>
<thead>
<tr>
<th>Microorganisms/Agents</th>
<th>NaOCl</th>
<th>CHX</th>
<th>Ca(OH)(_2)</th>
<th>HICA</th>
<th>alpha-mangostin</th>
<th>fluconazole</th>
<th>echinocandins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>gram positive aerobes</strong></td>
<td></td>
<td></td>
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<tr>
<td>Streptococci</td>
<td>++ (Heling et al., 2001; de Almeida Gomes et al., 2005)</td>
<td>V (Encan et al., 2004; Rôças and Siqueira, 2011)</td>
<td>++ (de Almeida Gomes et al., 2005; McBain et al., 2003)</td>
<td>(de Almeida Gomes et al., 2002; FIGUEIREDO et al., 2006; Estrela et al., 1998)</td>
<td>++ (Sakko et al., 2012)</td>
<td>+ (Tanungruang et al., 2013)</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>++ (de Almeida Gomes et al., 2005; Vianna et al., 2004)</td>
<td>V (Encan et al., 2004; Rôças and Siqueira, 2011)</td>
<td>++ (de Almeida Gomes et al., 2005; Vianna et al., 2004)</td>
<td>V (Encan et al., 2004; Rôças and Siqueira, 2011)</td>
<td>++ (Sakko et al., 2012)</td>
<td>+ (Pothisrat et al., 2010; Al-Massarani et al., 2013)</td>
<td>-</td>
</tr>
<tr>
<td>MRSA</td>
<td>++ (Lee et al., 2009; Altieri et al., 2013)</td>
<td>++ (Altieri et al., 2013)</td>
<td>ND</td>
<td>++ (Sakko et al., 2012)</td>
<td>++ (Koh et al., 2013)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Enterococci</td>
<td>V (Encan et al., 2004)</td>
<td>++ (Gomes et al., 2001, 2004, 2006)</td>
<td>V (Gomes et al., 2001; Vianna et al., 2004)</td>
<td>V (Gomes et al., 2001; Vianna et al., 2004)</td>
<td>- (Evans et al., 2002; Ballal et al., 2007)</td>
<td>+ (Koenigskolzig et al., 2015)</td>
<td>-</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>++ (Kubota et al., 2009)</td>
<td>+ (McBain et al., 2003)</td>
<td>V (FIGUEIREDO et al., 2006)</td>
<td>++ (Sakko et al., 2012)</td>
<td>+ (Gutierrez-Orozco et al., 2014)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>gram negative aerobes</strong></td>
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</tr>
<tr>
<td>Coliforms</td>
<td>++ (Zhang and Farahbakhsh, 2007)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>++ (McBain et al., 2003)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>ND</td>
<td>+ (Al-Massarani et al., 2013)</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>++ (DeQueiroz and Day, 2007; Small et al., 2007)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>++ (Moore et al., 2008)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>++ (Sakko et al., 2012)</td>
<td>+ (Al-Massarani et al., 2013)</td>
<td>-</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>++ (Radcliffe et al., 2004)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>++ (Vianna et al., 2004)</td>
<td>V (Rôças and Siqueira, 2011)</td>
<td>++ (Sakko et al., 2012)</td>
<td>+ (Al-Massarani et al., 2013; Asaavudip et al., 2014)</td>
<td>-</td>
</tr>
<tr>
<td>Candida</td>
<td>++ (de Almeida Gomes et al., 2005; Radcliffe et al., 2004)</td>
<td>++ (de Almeida Gomes et al., 2005; Vianna et al., 2004)</td>
<td>++ (de Almeida Gomes et al., 2002; Estrela et al., 1998)</td>
<td>(Nieminen et al., 2014a; Sakko et al., 2014)</td>
<td>++ (Koenigskolzig et al., 2009; Al-Massarani et al., 2013)</td>
<td>++ (Reboli et al., 2007)</td>
<td>++ (Kuhn et al., 2002; Choi et al., 2007; Bachmann et al., 2002)</td>
</tr>
<tr>
<td>Biofilms</td>
<td>++ (Lee et al., 2009; Altieri et al., 2013)</td>
<td>++ (Altieri et al., 2013)</td>
<td>+ (Upadhye et al., 2011)</td>
<td>++ (Nieminen et al., 2014a; Nieminen et al., 2014a)</td>
<td>ND</td>
<td>- (Mukherjee et al., 2003)</td>
<td></td>
</tr>
</tbody>
</table>

++ = good evidence of good activity; standards methodology (EUCAST or CLSI) employed and log reductions achieved
v = good evidence of good activity (in vivo study)
+ = evidence of some activity or non-standardised method
- = evidence of poor activity
ND = No data
1.8 Methods to study antimicrobial effects

The main methods used for in vitro screening for the efficacy of the novel antimicrobial substances are disk-diffusion and broth or agar dilution. In these, free-living (planktonic) cells in pure culture are exposed to known concentrations of the antimicrobial agents (Balouiri et al., 2016).

The disk diffusion susceptibility method is simple and useful for screening for the efficacy of known antimicrobial agents against a clinical isolate. The test is performed by spreading a bacterial/fungal inoculum thinly to the surface of the agar plate. Then, an antimicrobial disk with known amount of drug is placed on the inoculated agar surface. The drug diffuses into the agar developing a concentration gradient. Plates are normally incubated for 16–24 hours after which the zone of growth inhibition around each disk is measured. In addition to the susceptibility of the isolate, the diameter of the inhibition zone depends on the diffusion rate of the antimicrobial through the agar medium (Reller et al., 2009). The diffusion methods are sometimes used for preliminary screening of pure substances including alkaloids, flavanoids, terpenoids, etc. The advantages of this method are small amount of compound required and the possibility of testing up to six compounds against a single microorganism on one plate. However, these methods are not appropriate for testing lipophilic agents and cannot be used to determine the MIC (Rios et al., 1988).

The broth dilution method involves preparing a two-fold dilution of each tested antimicrobial (for example 1, 2, 4, 8, and 16 mg/L) into a liquid growth medium in test tubes (dilution method) or in 96-well plates or even smaller volumes (micro-dilution methods) and then inoculated with a standardised bacterial/fungal suspension (Jorgensen and Turnidge, 2015). Following overnight incubation, growth inhibition is assessed visually or spectrophotometrically. The lowest concentration of the antimicrobial that prevents growth of the target species represents minimal inhibitory concentration (MIC). The concentration of antimicrobial that inhibits 50% (MIC50) or 90% (MIC90) of growth are often determined and used to assess the potential clinical activity. This method is broadly used in clinical diagnostics (VITEK2) as well as in preliminary screening of potential new antimicrobial agents (Cuenca-Estrella et. al., 2010). The two main clinical diagnostic standards for susceptibility testing are the
American Clinical and Laboratory Standards Institute (CLSI) standard (Shryock, 2002; Wayne, 2009; Edition, 2012) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard; both are based on a 96-well plate microdilution method (Kahlmeter et al., 2006; Leclercq et al., 2013). In addition to defining the MIC, these methods can be used to define the minimal bactericidal concentration (MBC) or the minimum fungicidal concentration (MFC) by sub-culturing wells onto an agar plate (Balouiri et al., 2016). Susceptibility testing is also used for the regulatory approval of novel antiseptics and disinfectants; a representative number and spectrum of species and isolates is required for registration as a medicinal product via the EMA process (EMA, 2011).

The standard broth dilution methods require modifications when testing the activity of antimicrobial agents against biofilms (Donlan, 2001). The key differences are that instead of microbial suspensions pre-grown biofilms are exposed to the decreasing concentrations of antimicrobials, and that the growth inhibition is assessed by measuring biofilm metabolic activity or biomass. In general, 100 to 1000-fold or even higher concentrations are required (Pierce et al., 2008; Ramage et al., 2001c). Biofilms can be grown on coverslips or directly in the wells of polystyrene plates, and their maturity prior to antimicrobial exposure can be roughly assessed by bright-field and phase-contrast microscopy (Schlecht et al., 2015; Chevalier et al., 2017). Moreover, biofilms can be grown on pegs attached to 96-well plate lids (e.g. MBEC™ system). This assay is suitable for a large-scale susceptibility testing of antimicrobial activity against biofilms in both clinical and industrial settings (Bardouniotis et. al., 2001).

The cell viability within biofilms can be determined by scraping the microbial deposits formed onto solid supports followed by serial dilutions on appropriate agar plates for colony-forming units (CFU) count (Sousa et al., 2016; Barbosa et al., 2016; Matsubara et al., 2016; Sherry et al., 2016). However, the accuracy of this method and reproducibility of the results is often limited. Alternatively, crystal violet and colourimetric methods can be used to quantify in-well biomass formation (Montelongo-Jauregui et al., 2016; Xu et. al., 2016). The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, is commonly used to quantify biofilm metabolic activity (Park et al., 2014;
Matsubara et al., 2016). Moreover, the LIVE/DEAD® Biofilm viability kit (BacLight™, Invitrogen, Paisley, UK) can be used to visualise or quantify cells within biofilms; the difference in the green-fluorescent (SYTO9) and red-fluorescent (propidium iodide) nucleic acid stains allows differentiation between healthy cells (green) and cells with damaged membranes (red) (Shen et al., 2010; Standar et al., 2010). In addition, the dynamic interactions between antimicrobial agents and microbial strains and time required for bactericidal or fungicidal effect can be assessed in time-kill studies (Pfaller et. al., 2004). Molecular methods such as quantitative reverse transcription polymerase chain reaction (RT-qPCR) is suitably adapted for characterising the taxonomic and functional profile of multi-species biofilm models based on selected primers (Falsetta et al., 2014; Park et al., 2014; Thurnheer et al., 2016; Sherry et al., 2016; Yassin et al., 2016).

To simulate endodontic infections, dentine and root canal disinfection assays can be performed on standardised root blocks (Basrani et al., 2002; Sakko et al., 2017). Microorganisms are incubated in the root canal blocks and then exposed to the test substances. The sampling is done by drilling a defined thickness of the root canal wall off and washing the drill in broth which is then cultured onto agar plates. The quantification of the root canal samples can be performed by the CFU counting (Alves et al., 2013), spectrophotometric analysis (Sakko et al., 2017), as well as qPCR methods (Kim et al., 2013).

1.9 Summary

The main aetiological factor associated with pulpal and periapical diseases is the presence of microorganisms. The environment in the non-vital root canal system supports the growth of biofilms. These consist of bacteria or fungi or the combination of these. *E. faecalis* and *C. albicans* biofilms predominate in persistent endodontic infections as the interactions between these species support the progression of disease. The goal of endodontic treatment is to eradicate infection and to prevent microorganisms from re-infecting the root canal system. Root canal debridement using mechanical procedures effectively eradicates and kills some microorganisms, but others persist in the complex root canal systems and require chemical disinfectants. These procedures should be performed under aseptic conditions using rubber dam and sterile instruments, and include removal of failed restorations.
Chemical disinfection in endodontic procedures comprises of two steps. The first step is root canal irrigation. The standard root canal irrigant is sodium hypochlorite which not only possesses an antimicrobial effect, but also dissolves necrotic and vital organic tissue. However, concentrated sodium hypochlorite is highly toxic to surrounding tissues and if extruded into vital tissues it can cause severe side effects such as swelling and haematoma. CHX is an alternative root canal irrigant that also has a broad-spectrum antibacterial activity and substantivity but is also toxic to viable tissues and can cause allergic reactions. The second step of disinfection is placement of intra-canal inter-appointment medicaments. The ideal intra-canal medication would inhibit proliferation of microorganisms and eliminate them. Moreover, it should minimise ingress of an infection through a leaking restoration. Calcium hydroxide has these properties and induces hard tissue formation but resistance to it is common.

Interestingly, many natural products possess required properties for root canal irrigants and medicaments including the potential for elimination of pathogenic microorganisms in the root canal systems without the risk of cytotoxicity. Both HICA and alpha-mangostin are examples of natural products which are known to possess both properties, and thus have the potential to be used as alternatives in root canal disinfection. Further investigation of their properties and activity on pathogenic endodontic biofilms is required. To mimic the conditions in the root canals, biofilm models should be used and both bacterial and fungal species should be included.
1.10 Aims and objectives

The overall aim of this doctoral project was to investigate the antimicrobial properties of root canal medicaments on bacterial, fungal and multi-species biofilms. Novel and alternative antiseptic intra-canal medicaments were compared. The specific objectives were to:

1. Investigate the antimicrobial activity of HICA and alpha-mangostin against single species biofilms of *C. albicans* and *E. faecalis*.

   A. To determine the minimal inhibitory concentrations (MICs), the minimal fungicidal concentrations (MFCs), and/or the minimal bactericidal concentrations (MBCs) of each planktonic species in the multi-species biofilm after exposure to HICA and alpha-mangostin using a CLSI broth microdilution method (CLSI, 2008a; CLSI, 2009) in microtiter plates.

   B. To determine which ratio between *C. albicans* and *E. faecalis* should be used in the multiple-species biofilm model to mimic the clinical scenario by analysing growth curves.

   C. To determine the activity of HICA and alpha-mangostin on preformed *C. albicans* and *E. faecalis* biofilms *in vitro* using the XTT assays.

   D. To visualise the impact of HICA and alpha-mangostin on the *C. albicans* and *E. faecalis* biofilm structure using the bright field and fluorescence microscopy.

2. Investigate the antimicrobial activity of HICA and alpha-mangostin against multi-species biofilms.

   A. To determine the minimal inhibitory concentrations (MICs), the minimal fungicidal concentrations (MFCs), and/or the minimal bactericidal concentrations (MBCs) on each planktonic species in the multi-species biofilm after exposure to HICA and alpha-mangostin using a CLSI broth microdilution method (CLSI, 2008a; CLSI, 2009) in microtiter plates.

   B. To determine the activity of HICA and alpha-mangostin on preformed *C. albicans*, *E. faecalis*, *L. rhamnosus*, *S. salivarius* and *S. gordonii* mixed biofilms *in vitro* using the XTT assays.
C. To visualise the impact of HICA and alpha-mangostin on the C. albicans, E. faecalis, L. rhamnosus, S. salivarius and S. gordonii mixed biofilm structure using the bright field and fluorescence microscopy.

3. Investigate the antimicrobial activity of HICA and alpha-mangostin against multi-species biofilms in an ex vivo tooth model.

   A. To determine the time-kill assays of the planktonic C. albicans, E. faecalis, L. rhamnosus, and S. gordonii after exposure to HICA, alpha-mangostin, Calcicur® paste, and Odontopaste® paste.

   B. To determine the percent cell growth inhibition of multi-species biofilm after exposure to HICA, alpha-mangostin, Calcicur® paste, and Odontopaste® paste.

   C. To determine the viable cell counts, percent reduction, and bacterial/fungal load in dentin (CFU/mL) in multi-species biofilms after exposure to HICA, alpha-mangostin, Calcicur® paste, and Odontopaste® paste.


   A. To quantify the total and live cells, and to determine biofilm composition from experimentally infected root canal biofilms following exposure to HICA, alpha-mangostin, Calcicur® paste, and Odontopaste® paste by molecular (qPCR) methodologies.

   B. To determine relative cell growth (%) in experimentally-infected root canals after exposure to antimicrobials compared to 0.9% saline solution.
1.11 References


Yamazaki, Y. & Maeda, H. (1986). Enzymatic synthesis of optically pure (R)-(—)-mandelic acid and other 2-hydroxycarboxylic acids: Screening for the enzyme,


Chapter 2

Investigation of the antimicrobial activity of HICA and alpha-mangostin against the biofilms of *Candida albicans* and *Enterococcus faecalis*
2.1 Abstract

**Background:** Polymicrobial bacterial-fungal biofilms are common in a spectrum of infectious diseases including endodontic infections. Such biofilms resist antimicrobial treatment and alternative approaches are warranted. Disinfecting agents such as chlorhexidine (CHX) and sodium hypochlorite (NaOCl) are used as a root canal irrigants, but they can be toxic to human tissue. D,L-2-hydroxyisocaproic acid (HICA) is a highly biocompatible, bactericidal and fungicidal agent. It has also been shown to have activity against candidal biofilms. 1,3,6-Trihydroxy-7-methoxy-2,8-bis-(3-methyl-but-2-en-yl)-9H-xanthen-9-one (alpha-mangostin) has also been shown to possess antimicrobial activity against a wide range of microorganisms, but its efficacy against biofilms has not been studied.

**Aims:** The aim of this study was to determine the activity of HICA and alpha-mangostin on preformed *Candida albicans-Enterococcus faecalis* biofilms in vitro, and to visualise their impact on biofilm structure.

**Material/methods:** Single and dual species of *C. albicans* and *E. faecalis* (1:100 dual species ratio, respectively) biofilms were grown on polystyrene coverslips in RPMI for 48 hours at 37°C. Following this, the biofilms were exposed to 5% (w/v) HICA or 0.2% (w/v) alpha-mangostin for 24 hours. 2% (v/v) CHX and 2.5% (v/v) NaOCl were used as positive controls (five minutes exposure time) and RPMI as the negative control. After exposure, the metabolic activity of the biofilms was measured using the XTT assay, and biofilms were visualised using fluorescent BacLight™ LIVE/DEAD staining.

**Results:** All antimicrobial agents inhibited the metabolism of the biofilms compared to the RPMI control. NaOCl inhibited the metabolic activity of single and multi-species biofilms by at least 98%. Alpha-mangostin was significantly more active against all biofilms than HICA (p<0.001), and it was significantly more active against *E. faecalis* than *C. albicans* biofilms (p<0.001) by XTT assays. HICA and alpha-mangostin exposure reduced the number of cells in the *C. albicans* biofilms and no hyphae were observed. Exposure to both reagents reduced the number of viable cells in the biofilms as assessed by the BacLight™ LIVE/DEAD staining.

**Conclusion:** Both HICA and alpha-mangostin inhibited the metabolic activity of bacterial-fungal biofilms effectively. HICA is significantly less active against the bacterial biofilms than alpha-mangostin (p<0.001), but it is highly biocompatible. The
inhibitory effects of alpha-mangostin on biofilm metabolism are comparable to that of highly active but toxic NaOCl. Thus alpha-mangostin has potential as a novel antimicrobial agent and could be used to treat superficial infections or as an irrigant in root canal treatment.

2.2 Introduction

Root canal infections are polymicrobial biofilm-mediated diseases. This together with the complexity and variability of the root canal anatomy make disinfection of this system extremely challenging (Neelakantan et al., 2017). Moreover, the most important factor for failure of root canal treatment is microbial persistence (Siqueira, 2001). In endodontic treatment, biofilm removal is accomplished by a chemo-mechanical preparation. The specific instruments and disinfecting agents in the form of irrigants and/or intra-canal inter-appointment medicaments are used.

C. albicans is an opportunistic pathogen which colonises the gastrointestinal tract, upper respiratory tract and oral and vaginal mucosa in both of healthy and medically compromised individuals (Siqueira et al., 2004; Kim and Sudbery, 2011). In the oral cavity, C. albicans is mainly found at sites where endogenous oral commensal bacteria are also present. It only causes infection if its amount exceeds the tolerance of the underlying mucosae, or the host immune responses are locally or systemically impaired (Ghannoum et al., 2010; Rautemaa and Ramage, 2011). One of the major virulence factors of C. albicans is its ability to form biofilms (Costerton et al., 1999; Ramage et al., 2009).

E. faecalis is a Gram-positive facultatively anaerobic coccus which is frequently isolated from sites of oral infection including carious lesions, chronic periodontitis, and persistent apical periodontitis (Stuart et al., 2006; Zhu et al., 2010). E. faecalis can penetrate deep into dentinal tubules and adapt to the harsh environmental conditions (Rôças et al., 2004a). It is occasionally detected in primary endodontic infections (Gomes et al., 2008; Kaufman et al., 2005; Sassone et al., 2007) and it is a common finding in persistent endodontic biofilm infections (Bonten et al., 2001; Kaufman et al., 2005; Pinheiro et al., 2003; Rôças et al., 2004b). E. faecalis resist chemo-mechanical treatment and it also survives in nutrient-deficient conditions (Stuart et al., 2006; Kayaoglu and Ørstavik, 2004; Sedgley et al., 2005).
Mixed bacterial–fungal biofilms are always present in the oral environment including infected root canals (Ferrari et al., 2005). *E. faecalis* and *C. albicans* are the two most commonly recovered species in root canals undergoing re-treatment due to failure of the primary endodontic treatment and with persistent infection (Gopikrishna et al., 2006; Hong et al., 2013; Tennert et al., 2014). The complex structure of the biofilm allows some degree of interspecies metabolic interactions and commensal relationships to develop between the two populations (Romano and Kolter, 2005; Seneviratne et al., 2008). *C. albicans* is the most studied fungal pathogen in bacterial–fungal biofilms (Wargo and Hogan, 2006; Morales and Hogan, 2010; Shirliff et al., 2009).

Chemical disinfectants are used for both root canal irrigation and intra-canal medication between appointments. The main objective for irrigants used in endodontic treatment is to eradicate and kill the microorganisms from the root canal systems. Sodium hypochlorite is the standard root canal irrigant. In addition to its antimicrobial activity it also dissolves the necrotic and vital organic tissue and enables their eradication (Zehnder et al., 2002). Chlorhexidine (CHX) digluconate is also used as a root canal irrigant since it has broad-spectrum antibacterial activities against microorganisms found in infected root canals (Siqueira et al., 2007). In addition, CHX acquires the antimicrobial substantivity in the dentine medicated (Khademi et al., 2006; Carrilho et al., 2010; Gomes et al., 2013), the slowly released positively charged ions are adsorb into dentine and prevent microbial colonisation on the dentine surface up to four weeks (Khademi et al., 2006). However, CHX not dissolve pulp tissue whereby it cannot replace sodium hypochlorite (Haapasalo et al., 2010). An ideal intra-canal medication kills remaining microorganisms; moreover, it should minimise ingress of infection in case of a leaking restoration (Hargreaves, 2010). Calcium hydroxide (Ca(OH)₂) is the first choice as a root canal medicament: it has anti-bacterial effects due to its high pH (12.5–12.8) (Farhad and Mohammadi, 2005; Kim and Kim, 2014). Moreover, the bacterial products such as lipopolysaccharide are denatured and detoxified by calcium hydroxide (Tanomaru et al., 2003).

D,L-2-hydroxyisocaproic acid (HICA) is a leucine derivative and a normal, low-abundance constituent of human plasma as a result of leucine metabolism (Hietala et al., 1979). It is also found in human muscle and connective tissues (Guo et al., 2012; Mero et al., 2010). Recent studies show promising efficacy of HICA against
the growth of planktonically grown C. albicans (Sakko et al., 2014; Sakko et al., 2015), HICA also inhibits C. albicans hyphal growth and biofilm formation (Nieminen et al., 2014). At a concentration of 36,000 mg/L, HICA has broad-spectrum bactericidal activity against Gram-positive and Gram-negative bacteria (Sakko et al., 2012; Sakko et al., 2014). Thus, HICA may be considered as a potential agent for the topical treatment of bacterial-fungal biofilm infections (Nieminen et al., 2014).

Mangosteen (Garcinia mangostana Linn.) pericarp extracts have been shown to possess antimicrobial activity against a wide range of microorganisms (Nguyen and Marquis, 2011). The active components of mangosteen belong to a group of xanthone derivatives including 1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-enyl)xanthen-9-one (alpha-mangostin) which has the most potent antimicrobial activity (Chomnawang et al., 2005; Obolskiy et al., 2009; Suksamrarn et al., 2003). The planktonic MIC of alpha-mangostin for bacteria and fungi has been reported to range between 12,500-50,000 mg/L and 1,000-5,000 mg/L, respectively (Chin et al., 2008). There are no studies on the activity of the alpha-mangostin on bacterial or fungal biofilms.

In addition to colony counting, a metabolic assay (with XTT) is often used to quantify biofilm biomass indirectly. The metabolic activity is quantified spectrophotometrically. The XTT assay is based on the reduction of the tetrazolium salt of XTT in formazan by the succinate dehydrogenase system of the mitochondrial respiratory chain in cells (Park et al., 2014; Matsubara et al., 2016). The XTT assay is one of the routine laboratory methods when studying biofilms due to its simplicity, reproducibility, and moderate cost (Chevalier et al., 2017).

The aim of this study was to determine the activity of HICA and alpha-mangostin on preformed biofilms of C. albicans and E. faecalis in vitro. The hypothesis is that HICA and alpha-mangostin inhibit the growth and development of preformed C. albicans and E. faecalis biofilms.

2.3 Materials and methods

2.3.1 Study design

The planktonic growth of E. faecalis and C. albicans was followed and CFUs enumerated for up to 48 hours (Figure 2.1). The susceptibility of planktonic E. faecalis and C. albicans to HICA or alpha-mangostin (AM) was tested using a 96-
well plates microdilution method. Then, biofilms of *C. albicans, E. faecalis* or *C. albicans* and *E. faecalis* were grown on coverslips in 24-well plates for visualisation or in 96-well plates for metabolic activity assessments (XTT assays) in RPMI media for 48 hours (Figure 2.1). 1.0x10^6 cells/mL inoculum was used for the single species biofilms. For the dual species biofilm experiments, 1.0x10^6 cells/mL of *C. albicans* and 1.0x10^8 cells/mL of *E. faecalis* (1:100 ratio) was used. The growth conditions and microbial suspensions used were based on pilot experiments. Preformed biofilms were exposed to HICA and alpha-mangostin for 24 hours at pH 5.2. RPMI was used as a negative control while 2.5% sodium hypochlorite (NaOCl) and 2% chlorhexidine (CHX) were used as positive controls. The activity of these agents against the biofilms was assessed by measuring the biofilm metabolic activity using the XTT assay. Bright field and fluorescence microscopy were used to visualise biofilms. Differences between the parameters measured for biofilms exposed to HICA or alpha-mangostin and control conditions were compared statistically using one-way analysis of variance (one-way ANOVA). All biofilm experiments were done twice in triplicate. In total, 704 biofilms were used in the study.
Figure 2.1 Summary of the study design.

2.3.2 Media

RPMI-1640 (R0883, Sigma-Aldrich, Missouri, USA) with 2.0 g/L D-glucose and supplemented with sodium bicarbonate and L-glutamine, and buffered to pH 7.4 with morpholinepropanesulfonic acid (MOPS) (Oxoid, Basingstoke, UK) was filtered through a 0.22 µm membrane filter (Sigma-Aldrich, Missouri, USA) and stored at 4°C. The final concentration of RPMI-1640 used to prepare biofilms was single strength (1x) and double strength (2x) for single and dual-species biofilms, respectively.

2.3.3 Antimicrobial agents

A 5% (w/v) solution of D,L-HICA (TCI Europe, Zwijndrecht, Belgium) and a 0.2% (w/v) solution of alpha-mangostin (Sigma-Aldrich, Missouri, USA) were
prepared separately in 2xRPMI medium. The pH was adjusted to 5.2 for HICA and alpha-mangostin, then filtered through a 0.22 µm membrane filter. HICA was prepared fresh each time, alpha-mangostin was preparations and stored at 4°C.

2.3.4 Microbial isolates and culture conditions

Two isolates of *E. faecalis* were used in this experiment. The clinical isolate of *E. faecalis* (T 75359) was derived from a root canal and identified using conventional biochemical methods and 16S sequencing (Sakko et al., 2015). *E. faecalis* ATCC 29212 (urine isolate) was used as a reference strain (Sakko et al., 2015). Bacterial isolates were cultured on blood agar (BA, Oxoid, Basingstoke, UK) at 37°C for 24 hours. One colony was then inoculated into 20 mL LB broth (Lennox, Massachusetts, USA) and grown overnight at 37°C in a shaking incubator (250 rpm maximum). The cells were harvested by centrifugation (4,500 rpm for 5 minutes), washed twice with sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, Missouri, USA) and then re-suspended in 1xRPMI-1640. The cell density was measured by using a spectrophotometer and adjusted to 0.1 OD at 600 nm, which was equivalent to 1.0x10^8 cells/mL.

Two isolates of *C. albicans* were used in this experiment. The clinical isolate of *C. albicans* (D 17) was derived from a root canal and identified using conventional biochemical methods and 16S sequencing (Sakko et al., 2014). *C. albicans* ATCC 10231 (bronchial isolate) was used as a reference strain (Sakko et al., 2014). *C. albicans* was cultured on Sabouraud Dextrose (SAB) agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 48 hours before being used to check viability (by plating) and purity (by microscope). One colony was then inoculated into 20 mL yeast peptone dextrose (YPD) broth (Melford, Ipswich, UK) and grown overnight at 37°C in the shaking incubator (250 rpm maximum). The cells were harvested by centrifugation (3,000 rpm for 5 minutes), washed twice with sterile phosphate-buffered saline (PBS) and then re-suspended in 1xRPMI-1640. To produce the standardised biofilm inoculum, the cell density was measured by using a Neubauer haemocytometer and adjusted to 1.0x10^6 cells/mL. The cell counts were confirmed by plating technique.

For the mixed biofilms, the enterococcal and candidal suspensions were prepared and tested in 1:1, 1:2 and 1:100 *C. albicans: E. faecalis* ratios. The dual-
species biofilms were prepared from a stock of doubly concentrated *E. faecalis* and *C. albicans* cells in 2xRPMI.

### 2.3.5 Susceptibility testing

Broth microdilution methods were used to determine the susceptibility of the planktonic *C. albicans* isolates (CLSI, 2008a) and *E. faecalis* isolates (CLSI, 2009) to HICA and alpha-mangostin with a modification of how results were derived. The minimum inhibitory concentration (MIC) was read spectrophotometrically after 24 hours of incubation instead of visually. The MIC was determined as the lowest concentration of drug that resulted in significant inhibition of growth.

HICA was diluted from a freshly made 200,000 mg/L aqueous solution, pH 5.2, into RPMI broth while alpha-mangostin was diluted from a freshly made 64,000 mg/L aqueous solution into RPMI broth for the 2-fold dilutions (range 200,000 to 1.6 mg/L for HICA and 64,000 to 0.02 mg/L for alpha-mangostin) as previously described (Sakko et al., 2012; Sakko et al., 2014; Kaomongkolgit et al., 2013). Commercial CHX at 20,000 mg/L (Corsodyl®, Glaxo Smith Kline, Middlesex, UK) and sodium hypochlorite (NaOCl, Davis Schottlander & Davis Ltd., Hertfordshire, UK) at 25,000 mg/L were used as comparators. RPMI medium was used as a negative control to determine the maximum microbial growth.

Pure cultures of *C. albicans* were grown aerobically on SAB at 37°C for 48 hours while pure cultures of *E. faecalis* were grown on BA at 37°C for 48 hours aerobically. An aqueous stock solution of DL-HICA (pH 5.2) was used. RPMI adjusted to pH 5.2 was used as the culture medium to support the growth of all species. The final density of 1x10⁶ cells/mL of candidal and 1x10⁸ cells/mL of enterococcal cells was used. The cell candidal density was estimated by haemocytometer, and enterococcal cell density spectrophotometrically. Cell densities were confirmed by quantitative culture.

For susceptibility testing, the planktonic microbial suspensions (20 μL) were added to broth (180 μL) in 96-well cell-culture microtiter plates (F96 MicroWell Plates, NUNC, ThermoFisher Scientific, Leicestershire, UK). The growth of *C. albicans* and *E. faecalis* species was determined spectrophotometrically (OD490). The minimum inhibitory concentration (MIC) was the lowest concentration, which
reduced the optical density by 50% (IC$_{50}$) and 90% (IC$_{90}$) in comparison to the drug-free controls at 24 hours. The tests were done with three replicate wells and repeated. The microbiocidal activity of HICA and alpha-mangostin was tested after MICs were determined by streaking 20 μL of the test suspensions from representative wells on agar for 48 hours. Microbial growth was recorded as present or absent. The results of the microbiocidal activity were shown as minimum bactericidal or fungicidal concentration (MBC/MFC).

2.3.6 Growth curve

To understand the planktonic growth pattern of each isolate their growth curves were established. The organisms were incubated with an adjusted amount of the cells equal to 1.0x10$^6$ cells/mL in brain heart infusion (BHI; Difco Laboratories) medium in 96-well round bottom plates. For the dual species experiments, 1:1 and 1:100 ratios of C. albicans:E. faecalis were prepared. Cells were harvested after 0, 6, 12, 24, 36, and 48 hours without addition of new nutrients. Ten-fold serial dilutions were made into PBS, and then 10 μL of each dilution was spread on BHI agar plates. Haemocytometer and cell plating were used to determine cell counts at each time point. These procedures were done in triplicate.

2.3.7 Biofilm growth and treatment

Biofilms were grown in 24-well plates for visualisation and in 96-well plates for susceptibility testing. For the 24-well plate protocol, C. albicans, E. faecalis, and dual biofilms were grown on Thermanox coverslips (Thermo Scientific Nunc, Illinois, USA) in RPMI at pH 7.4 without shaking (Corning CoStar, North Carolina, USA) for 48 hours at 37°C. The biofilms were then exposed to 5% (w/v) HICA, 0.2 % alpha-mangostin, or RPMI at pH 5.2 for another 24 hours at 37°C. The method for preparing biofilms in 96-well plates was identical to that for 24-well plates; 96-well plates can be used for direct analysis of the biofilms in situ without Thermanox coverslips.

2.3.8 Measuring the metabolic activity of fungal, bacterial, and mixed biofilms with XTT reagent

XTT assay is a colourimetric assay for determining cell metabolic activity (Kuhn et al., 2003). The XTT solution was prepared by adding 1 mM menadione
solution into 200 µL of saturated XTT (Nieminen et al., 2014). Before analysis, biofilms were washed with sterile PBS. Then 200 µL of XTT-menadione solution was added into separate well in 96-well plates, or 1 mL to 24-well plates. The plates were incubated in the dark at 37°C for 2 hours. After incubation, 100 µL of the XTT supernatant was transferred into separate wells of a fresh 96-well plate and the colourimetric changes were measured spectrophotometrically (BMG Labtech, UK) at 490 nm.

2.3.9 Biofilm visualisation and quantification

The pre-grown biofilms were washed once with PBS before being stained with BacLight™ LIVE/DEAD viability kit (Fisher Scientific, Leicestershire, UK) according to the manufacturer’s instructions; the stain contained a 1:1 ratio of SYTO9 and propidium iodide (PI). Biofilms were transferred to microscope slides, covered with coverslips and examined under bright field and fluorescence microscopy (Nikon Eclipse 80i). To detect PI fluorescence, the excitation filter was 532 nm and the emission 635 nm (Tx Red). To detect SYTO9 fluorescence, excitation filter was 483 nm and emission 503 nm (FITC). The PI and SYTO9 images were quantified using Image-Pro Plus 5.1 (Media Cybernetics, USA). After staining, all specimens were analysed by epifluorescence microscopy using a 6,000-fold magnification (Axioskop II, Zeiss; Oberkochen, Germany). The number of cells was counted in ten randomly selected non-overlapping microscopic ocular grid fields (0.0156 cm² grid area). Two different light filters were used for discrimination of dead and viable cells; Etbr filter was used for the visualisation of non-viable cells: BP 546/12, FT 580, and LP 590; and FDA filter for viable cells: BP 450–490, FT 510, and LP 515. Moreover, the replicate sets of control biofilms and those exposed antimicrobials were assessed for cell viability by culture of serial dilutions on agar plates and colony counting.

2.3.10 Statistical analysis

GraphPad Prism (version 7.03; La Jolla, CA, USA) was used for data analysis, to represent the data graphically and provide statistical analysis. One-way Analysis of Variance (ANOVA) was used to investigate significant differences between independent groups of data which followed normal Gaussian distribution (differences between HICA and alpha-mangostin treated biofilms compared to control conditions). To analyse the multiple comparisons of the data, a Bonferroni
correction was applied to the \( p \) value. Statistical tests were interpreted at the 5% significance level.

2.4 Results

2.4.1 Planktonic susceptibility testing

The planktonic susceptibility testing showed that both HICA and alpha-mangostin inhibited the growth of *E. faecalis* in a dose-dependent manner (Table 2.1). Both HICA and alpha-mangostin were more active against the *E. faecalis* isolates than the *C. albicans* isolates. At 24 hours, the HICA IC\(_{90}\) for the most susceptible *E. faecalis* isolate was 1,600 mg/L, and for the two *C. albicans* isolates were 50,000 mg/L. HICA was cidal against all tested isolates at 50,000 mg/L. The alpha-mangostin IC\(_{90}\) was 0.5 mg/L for both *E. faecalis* isolates and 125 mg/L for both *C. albicans* isolates. Alpha-mangostin was fungicidal against both *C. albicans* isolates at 1,000 mg/L concentration, while it was bactericidal against both *E. faecalis* isolates at 2 mg/L concentration (Table 2.1). Alpha-mangostin activity was improved by reduction of the pH from 7.4 to 5.2, as the IC concentrations were lower at pH 5.2 (Table 2.2).

**Table 2.1** The activity of the HICA and alpha-mangostin against *E. faecalis* and *C. albicans* isolates in acidic conditions.

<table>
<thead>
<tr>
<th>Organism and phenotype</th>
<th>Designation</th>
<th>Source</th>
<th>HICA (pH =5.2)</th>
<th>alpha-mangostin (pH =5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC(_{90}) (mg/L)</td>
<td>IC(_{90}) (mg/L)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>ATCC 10231</td>
<td>Man with broncho mycosis</td>
<td>50,000</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>D 17</td>
<td>Root canal</td>
<td>12,500</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>Urine</td>
<td>100</td>
<td>1,600</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>T 75359</td>
<td>Root canal</td>
<td>800</td>
<td>3.2</td>
</tr>
</tbody>
</table>

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; IC\(_{50/90}\), concentration that inhibits 50% and 90% of the growth, respectively.
Table 2.2 The activity of the alpha-mangostin against *E. faecalis* and *C. albicans* isolates in neutral and acidic conditions.

<table>
<thead>
<tr>
<th>Organism and phenotype</th>
<th>Designation</th>
<th>Source</th>
<th>alpha-mangostin (pH =7.4)</th>
<th>alpha-mangostin (pH =5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC_{50} (mg/L)</td>
<td>IC_{90} (mg/L)</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
<td>Man with broncho mycosis</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>D 17</td>
<td>Root canal</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC 29212</td>
<td>Urine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>T 75359</td>
<td>Root canal</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; IC_{50/90}, concentration that inhibits 50% and 90% of the growth, respectively.

2.4.2 Growth curve

The planktonic growth of *E. faecalis* and *C. albicans* followed a similar pattern; the amount of all planktonic living cells increased exponentially during the first 6 hours of incubation (exponential growth phase; Figure 2.2). Between 6 and 36 hours the amount of living cells remained stable (stationary phase) and decreased after 36 hours (starvation/death phase). This was confirmed by using either of the two cell counting techniques, haemocytometer and CFU counts, but the former gave 3.36-15 times higher values. From the dual-species analysis, both 1:1 and 1:100 *C. albicans* and *E. faecalis* ratio showed that the growth of neither was affected by the presence of the other (Figure 2.3). The 1:100 ratio was selected for the biofilm stage as this reflects better the situation in root canal infections (Sen et al., 1995; Peciuliene et al., 2001; Molander et al., 1998).
Figure 2.2 The growth of *E. faecalis* and *C. albicans* individually and in the presence of each other enumerated by CFU counting. Clinical isolates (A, B), reference strains (C, D), *E. faecalis* and *C. albicans* ratio 1:1 (left, A, C) 1:100 (right, B, D). Results of two independent experiments done in triplicate are presented.
2.4.3 HICA and alpha-mangostin have a significant inhibitory effect on biofilm metabolic activity

Exposure to antimicrobial agents reduced the metabolic activity of all pre-grown biofilms significantly (p<0.0001). Sodium hypochlorite inhibited the metabolic activity of both single and mixed species biofilms by 99.8% (Figure 2.3 and 2.4). There was no significant difference in the activity of the reagents against reference and clinical strain biofilms (Figure 2.3 A, B) (p=0.9793). The percent inhibition of metabolic activity in exposed to HICA is summarised in Table 2.3. Interestingly, HICA inhibited the metabolic activity of the dual-species *C. albicans* and *E. faecalis* biofilms more than that of the single species biofilms (p<0.0001) (Figure 2.3 A, B).

Table 2.3 Inhibition of biofilm metabolic activity after 24-hour exposure to the HICA and alpha-mangostin (AM) compared to the negative control (RPMI). Experiments were performed twice in triplicate.

<table>
<thead>
<tr>
<th>Agents/ Biofilms</th>
<th>C. albicans ATCC 10231</th>
<th>C. albicans D17</th>
<th>E. faecalis ATCC 29212</th>
<th>E. Faecalis T 75359</th>
<th>1:100 C. albicans ATCC 10231: E. faecalis ATCC 29212</th>
<th>1:100 C. albicans D17: E. faecalis T 75359</th>
</tr>
</thead>
<tbody>
<tr>
<td>HICA (pH=5.2)</td>
<td>41.8% (range 39.5-43.5%)</td>
<td>41.6% (range 40.4-43.4%)</td>
<td>61.6% (range 60.3-63.0%)</td>
<td>68.1% (range 67.1-69.1%)</td>
<td>80.2% (range 78.6-81.5%)</td>
<td>79.5% (range 77.5-81.1%)</td>
</tr>
<tr>
<td>AM (pH=5.2)</td>
<td>71.7% (range 70.1-72.2%)</td>
<td>76.9% (range 75.5-77.8%)</td>
<td>91.0% (range 89.9-92.0%)</td>
<td>93.5% (range 93.0-94.3%)</td>
<td>91.3% (range 91.0-91.7%)</td>
<td>96.9% (range 96.5-97.1%)</td>
</tr>
</tbody>
</table>

Alpha-mangostin was significantly more active against all biofilms than HICA (p<0.0001). It was also found to be 20% more active against *E. faecalis* than *C. albicans* biofilms (Figure 2.3 and 2.4). The percentage of the inhibition of biofilm metabolic activity after exposure to alpha-mangostin is shown in Table 2.3. There was no statistically significant difference in the metabolic activity of *E. faecalis* after exposure to alpha-mangostin or chlorhexidine control (Figure 2.3 A, B) (p=0.921). Alpha-mangostin inhibited the metabolic activity of the dual-species *C. albicans* and *E. faecalis* biofilms better than single species biofilms (p<0.0001) (Figure 2.3 A, B). Alpha-mangostin activity was further improved by lowering the pH to 5.2 (Figure 2.4).
Figure 2.3 Inhibition of metabolic activity in biofilms (A, clinical isolates, B, reference isolates) after 24-hour exposure to the antimicrobials compared to the negative control measured by XTT assay. Results of two independent experiments done in triplicate are presented (**p<0.0001: one way-ANOVA with a Bonferroni correction).
Figure 2.4 Inhibition of metabolic activity in biofilms after 24-hour exposure to alpha-mangostin in acidic and neutral conditions compared to the negative control measured by XTT assay. Results of two independent experiments done in triplicate are presented (**p<0.0001, ns= not significant: one way- ANOVA with a Bonferroni correction).

2.4.4 Visualisation and quantification of biofilms

Biofilms were intact prior to exposure to antimicrobials reagents (Figure 2.5-2.7). Some pseudo-hyphae were observed in the 48-hour *C. albicans* biofilms. In the untreated biofilms, 93.0% of candidal cells, 99.4% of enterococcal cells, and 98.6% of cells in dual-species biofilms were viable according to LIVE/DEAD staining.

HICA and alpha-mangostin exposure reduced the number of viable cells in the single species *C. albicans* biofilms. After exposure to HICA, 52.3% of the cells were viable, and after exposure to alpha-mangostin, 48.1% of cells were viable following staining. Exposure to either HICA or alpha-mangostin prevented candidal hyphal formation (Figure 2.5; panels c,d,e,f). After CHX exposure, 89.2% of cells in
candidal biofilms stained viable (Figure 2.5; panels g,h). However, the CHX markedly reduced the number cells in biofilms by 5-Log CFU/mL (Figure 2.8).

There were more dead cells in enterococcal biofilms compared to candidal biofilms after exposure to HICA, alpha-mangostin, or CHX (Table 2.4). In the dual-species biofilms, the presence of Candida appeared to improve the survival of the enteroccal cells following HICA or alpha-mangostin exposure (Figure 2.7; panel c,d,e,f). Interestingly, candidal hyphal structures were clearly visible in the dual-species biofilms after exposure to CHX although CHX treatment reduced the number of persisting cells in these dual-species biofilms (Figure 2.7; panels g,h; Figure 2.8).

Table 2.4 Quantification of live and dead cells of control biofilms, and biofilms after exposure to HICA, alpha-mangostin (AM), and chlorhexidine (CHX). The quantification was done by LIVE/DEAD staining.

<table>
<thead>
<tr>
<th>Agents/ Biofilms</th>
<th>Candidal biofilms</th>
<th>Enterococcal biofilms</th>
<th>Dual-species biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Live cells</td>
<td>% Dead cells</td>
<td>% Live cells</td>
</tr>
<tr>
<td>Control</td>
<td>93.0</td>
<td>7.0</td>
<td>99.4</td>
</tr>
<tr>
<td>HICA</td>
<td>52.3</td>
<td>47.7</td>
<td>34.8</td>
</tr>
<tr>
<td>AM</td>
<td>48.1</td>
<td>51.9</td>
<td>25.9</td>
</tr>
<tr>
<td>CHX</td>
<td>89.2</td>
<td>10.8</td>
<td>88.8</td>
</tr>
</tbody>
</table>
Figure 2.5 Impact of HICA and alpha-mangostin on candidal biofilms shown on the hyphal formation and morphology of C. albicans D 17 by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e–f), and comparator (chlorhexidine digluconate; g-h).
Figure 2.6 Impact of HICA and alpha-mangostin on enterococcal biofilms shown on the morphology of *E. faecalis* T 75379 by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (chlorhexidine digluconate; g-h).
Figure 2.7 Impact of HICA and alpha-mangostin on candidal and enterococcal biofilms. The morphology is shown by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (chlorhexidine digluconate; g-h).
After exposure to HICA, alpha-mangostin, or CHX, the number of CFU/mL was reduced by 3-Log and the inhibitory activity of alpha-mangostin and CHX was comparable (Figure 2.8).

**Figure 2.8** Quantification of viable cells (CFU/mL) of control biofilms, and biofilms after exposure to HICA, alpha-mangostin (AM), and chlorhexidine (CHX) by culture. The mean was presented in triplicate (**p<0.0001: one way- ANOVA with a Bonferroni correction).
2.5 Discussion

The results of the present study showed that HICA and alpha-mangostin possess anti-metabolic activity against *C. albicans*, *E. faecalis* and dual species *C. albicans-E. faecalis* biofilms. This is the first study reporting the effectiveness of HICA against *E. faecalis* and dual species *C. albicans-E. faecalis* biofilms, and the first report on anti-biofilm activity of alpha-mangostin. Due to the good biocompatibility and safety profile of HICA (EFSA; 2012), it could also be suitable for the treatment of infected body cavities, sinuses as well as dental root canals.

HICA is a promising agent for the topical treatment of superficial fungal, bacterial or mixed microfloral infections. Interestingly, the planktonic MICs reported here differed from those previous published for these compounds and isolates. Sakko et. al. reported 4,500 mg/L IC$_{50}$ of HICA for the same *E. faecalis* isolates (T 75359) (Sakko et. al., 2012), whereas this thesis reported 800 mg/L. On the other hand, the candidal IC results were higher than previously published: 18,000 mg/L IC$_{50}$ of HICA for *C. albicans* (D 17) has been reported (Sakko et. al., 2014) and this study reported 50,000 mg/L. Interestingly, alpha-mangostin, was found to be significantly more active than HICA against all biofilms tested based on the metabolic assay results and viability staining. Therefore, it appeared that alpha-mangostin could be used at much lower concentrations than HICA. Also, as it is active at neutral pH, not the case for HICA, it could be suitable for managing a boarder range of infections topically including root canal infections. However, the anti-biofilm activity of alpha-mangostin was further improved by reducing the pH as described previously for planktonic bacteria (Nguyen and Marquis, 2011). Unlike HICA, the mode of action of alpha-mangostin is known: it disrupts the cytoplasmic membrane of microorganisms (Koh et al., 2013) but less is known about its biocompatibility (Kaomongkolgit et al., 2009).

This study showed that the currently used root canal irrigants (NaOCl and CHX) had the ability to remove or disrupt biofilms, and that the remaining cells or persisting cells were killed *in situ*. All antimicrobial agents reduced survival of the cells significantly. The proteolytic NaOCl removed and killed cells within biofilms effectively, it dissolved all biofilms as assessed by viability staining. CHX also disrupted biofilms effectively with few survivor cells detected by culture, but viability
staining showed high proportion of viable cells. This can be partial due to non-viable cells becoming detached and many viable cells remaining. It is also possible that CHX modified the microbial cell membranes and leading to non-specific binding of the Syto9 (Stiefel et al., 2015).

The composition of the growth medium has a major effect on the in vitro biofilm architecture, protein expression profiles and the antifungal susceptibility (Kucharíková et al., 2011). Two growth media were compared in pilot experiments: thioglycollate medium was used to create a nutrient-rich environment and RPMI medium was used to mimic a physiological environment. The D-glucose concentration in RPMI medium was 2,000 mg/L and in thioglycollate broth 5,500 mg/L. These are higher than the physiological concentration normally found in the human body (700-1,000 mg/L) (Tirosh et al., 2005). As biofilm growth and maturation is enhanced by the presence of glucose, hyper-glycemic media are commonly used in biofilm studies (Coenye et al., 2010), it is important that the in vitro model used mimics the conditions in vivo at the site of infection as far as possible. We used RPMI medium without additional D-glucose throughout the biofilm experiments to achieve this.

For the dual-species experiments, 1:100 ratio for C. albicans and E. feacalis was selected as the growth rates of both species remained the same as in single species biofilms. Moreover, there are typically more bacteria than yeast cells in the infected root canal systems (Gomes et al., 2004). Both haemocytometer and CFU counting techniques were used to count cells at different time points. The haemocytometer technique provides immediate results and the CFU counting technique was used to confirm the cell counts and the number of viable cells at any time point.

One of the limitations of the study was that only two isolates of C. albicans and E. feacalis were used. However, both clinical and reference isolates were included in case. There were differences between the isolates with respect to metabolic activity, susceptibility profiles as well as the ability to form biofilms (Tumbarello et al., 2012). The findings in this study require further confirmation with a significantly higher number of isolates (more than hundred isolates of each species) including a representative number of isolates susceptible or resistance to one or more
antimicrobial agents examined for registration of HICA and alpha-mangostin as medicinal product under EMA process (EMA, 2011). Importantly, this work would be beyond the aims of this study. Secondly, only two species were included in the biofilm model although oral infections typically involve multi-species biofilms (Paju et al., 2007). However, persisting root canal infections often have only few dominant species (Lin et al., 2011; del Carpio-Perochena et al., 2015). Nevertheless, in the future, the antimicrobial activity of HICA and alpha-mangostin should be studied against more diverse biofilms. Finally, the possible interactions between alpha-mangostin and root canal substances (such as dentine and hydroxyapatite) have not been tested. This topic should be addressed in further studies in order to evaluate the potential of the agent as an inter-appointment intra-canal medicament in endodontics.

2.6 Conclusion

The impact of HICA and alpha-mangostin on C. albicans, E. faecalis, and dual-species C. albicans-E. faecalis biofilms showed that they have the potential to be developed into novel antimicrobial inter-appointment medications for the treatment of root canal infections. Further studies using multi-species infected root canal models and clinical trials are required before the clinical use of HICA and alpha-mangostin can be recommended.
2.7 References


different conditions and their susceptibility to caspofungin and anidulafungin. *Journal of medical microbiology*, 60(9), 1261-1269.


prenylated xanthones from the fruits of Garcinia mangostana. *Chemical and pharmaceutical bulletin*, 51(7), 857-859.


Chapter 3
Investigation of the antimicrobial activity of HICA and alpha-mangostin against multi-species biofilms
3.1 Abstract

**Background:** The multi-species nature of biofilms in infectious diseases makes disinfection challenging. Biofilm removal is best accomplished by a chemomechanical debridement. Chlorhexidine (CHX) and sodium hypochlorite (NaOCl) are disinfecting agents of choice but they can be toxic to human tissues. HICA (D,L-2-hydroxyisocaproic acid) is a by-product of leucine metabolism and can be detected in human tissues and plasma. It is a bactericidal and fungicidal agent. Alpha-mangostin (1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-enyl)xanthen-9-one) has also been shown to possess antimicrobial activity against a wide range of microorganisms. However, there are no studies on its activity on bacterial or fungal biofilms. Moreover, the activity of HICA or alpha-mangostin has not been tested against mixed species bacterial-fungal biofilms.

**Aims:** To determine the activity of HICA and alpha-mangostin on preformed bacterial-fungal multi-species biofilms *in vitro*, and to ascertain their impact on biofilm structure and viability.

**Methodology:** Minimal inhibitory concentrations (MICs) for HICA and alpha-mangostin against planktonic cultures of *Candida albicans*, *Enterococcus faecalis*, *Lactobacillus rhamnosus*, and *Streptococcus gordonii* or *Streptococcus salivarius* were determined using a standard broth microdilution method. Single and multi-species (all species 1:1:1:1) biofilms were grown on polystyrene cover slips in RPMI for 48 hours. The biofilms were then exposed to 5% (w/v) HICA or 0.2% (w/v) alpha-mangostin for 24 hours. These concentrations were selected based on pilot experiments and the solubility of these compounds. 2% (v/v) CHX and 2.5% (v/v) NaOCl were used as comparators, and RPMI as the negative control. The metabolic activity of the biofilms after exposure was measured using metabolic (XTT) assays, and the biofilms were visualized and quantified using fluorescent BacLight™ LIVE/DEAD staining.

**Results:** HICA was cidal against planktonic bacteria and *Candida* at 50,000 mg/L, while 8 mg/L of alpha-mangostin was cidal against planktonic bacteria and 1,000 mg/L for *Candida*. Both HICA and alpha-mangostin were most active against *L. rhamnosus* biofilms (metabolism inhibited by 98% and 99%, respectively) and least active against *Candida* biofilms (metabolism inhibited by 42% and 78%, respectively). Alpha-mangostin demonstrated better activity against multi-species
biofilms than HICA (93% vs 46% inhibition) (p<0.001). NaOCl inhibited the metabolic activity of single and multi-species biofilms by 98%. HICA and alpha-mangostin exposure reduced the number of cells in C. albicans biofilms and no hyphae were observed. Exposure to HICA or alpha-mangostin reduced the number of viable cells in the biofilms as assessed by the BacLightTM LIVE/DEAD staining.

**Conclusions:** Both HICA and alpha-mangostin inhibited the metabolic activity of bacterial-fungal biofilms effectively. The anti-biofilm activity of alpha-mangostin was comparable to that of the highly active but toxic NaOCl and thus has potential as a novel agent for endodontic therapy. HICA was less active against the biofilms than alpha-mangostin, but due to its high biocompatibility it also has potential but in the treatment of fractured or perforated root canals.

**3.2 Introduction**

The colonisation of microorganisms in a body site such as the root canal space is either as planktonic single cells or attached to each other and/or to the root canal walls as a biofilm. A biofilm is a community of microorganisms embedded in an extracellular polysaccharide matrix and typically attached on various surface structures in nature (Siqueira and Rôças, 2008) but can also adhere to a cavity as non-surface associated microcolonies (Loussert et al., 2010; Kernien et al., 2017). While planktonic microorganisms can be eradicated easily by a variety of disinfection methods, biofilms remain a major challenge (Haapasalo et al., 2005; Ricucci and Siqueira, 2010; Siqueira Jr et al., 2014). Apical periodontitis is an inflammatory reaction of periradicular tissues caused by a microbial infection in the root canal (Marton and Kiss, 2000). The microorganisms in the necrotic root canal system colonise mostly as biofilms (Rocha et al., 2008; Mohammadi et al., 2013) whereas successful endodontic treatment will depend on the effective elimination of such biofilms. Multi-species bacterial–fungal biofilms are commonly found in the oral environment including infected root canals (Ferrari et al., 2005) and they are difficult to treat with antibiotics and antifungal agents (Baena-Monroy et al., 2004).

The complex structure of the biofilm allows some degree of interspecies cooperation to develop between the populations, the specific interspecies and intra-species interactions in biofilms determine their organisation and cell growth (de Paz, 2007). There are metabolic interactions between microorganisms in biofilms,
including mutualistic and commensal relationships (Romano and Kolter, 2005; Seneviratne et al., 2008). There are physical interactions between *C. albicans* and biofilm-forming pathogenic bacteria and surface polysaccharides play an important role in the colonisation of bacterial biofilms by *C. albicans* and *vice versa*. Bacteria which produce a glycocalyx are able to adhere better to *C. albicans* biofilms. In contrast, the fungus cannot attach as easily to preformed biofilms of glycocalyx-producing bacteria (El-Azizi et al., 2004). The survival of *C. albicans* in the host requires a niche established within the multi-species communities of bacteria. Intermicrobial binding (coaggregation or coadhesion) between *C. albicans* and oral bacteria is important for *C. albicans* colonisation and its persistence within complex microbial biofilms (Cannon and Chaffin, 2001; Douglas, 2003).

Studies on root canal infections using various techniques have identified a selected number of persistent bacterial species. Gram-positive facultative anaerobic bacteria including enterococci, streptococci, lactobacilli, and actinomycetes are amongst the most common findings (Chavez De Paz et al., 2003; Rôças et al., 2004a). The development of *in vitro* multi-species biofilm models is challenging and the interactions between species in root canal biofilms is not yet fully explained. Single-species models are used in many biofilm studies in endodontics (Arias-Moliz et al., 2009; Arias-Moliz et al., 2010; Dunavant et al., 2006). Although some multi-species models have been used, the species composition has not considered (Shen et al., 2010; Shen et al., 2011). There are studies using four clinical bacterial strains isolated from infected root canals, *Actinomyces naeslundii*, *L. salivarius*, *S. gordonii*, and *E. faecalis* (Chávez de Paz et al., 2015; de Paz, 2012), in which stable and reproducible biofilm communities were formed in a mini-flow cell system (de Paz, 2012). Moreover, different isolates of *E. faecalis* can interact either synergistically or antagonistically with other species of root canal biofilms: the detachment of neighbouring bacteria and the level of tissue damage that occurs follows the production of different levels of *E. faecalis* proteases (Chávez de Paz et al., 2015).

To eradicate and kill the microorganisms from root canal systems, irrigation and intra-canal medicaments are used as chemical disinfectants. Sodium hypochlorite (NaOCl) is the standard root canal irrigant because of its antimicrobial activity. Moreover, sodium hypochlorite can dissolve necrotic and vital organic tissue (Zehnder et al., 2002). Chlorhexidine (CHX) digluconate is also used as a root canal irrigant; it has broad-spectrum antibacterial activities against microorganisms found
in infected root canals (Siqueira et al., 2007). Intra-canal medicaments are used to manage the root canal infection. The ideal properties of medication are to inhibit proliferation and further eliminate remaining microorganisms; moreover, it should minimise ingress of infection via a leaking restoration (Hargreaves, 2010). Calcium hydroxide (Ca(OH)$_2$) is the first choice as a root canal medicament; it has antibacterial effects due to its high pH (12.5-12.8) (Farhad and Mohammadi, 2005). Moreover, bacterial products such as lipopolysaccharide are denatured and detoxified by calcium hydroxide (Tanomaru et al., 2003).

D,L-2-hydroxyisocaproic acid (HICA) is produced by *Lactobacillus* species and is found in human plasma as a result of leucine metabolism (Hietala et al., 1979). HICA has antimicrobial activities against bacteria (Sakko et al., 2014; Sakko et al., 2012), and pathogenic fungi including several *Candida* and *Aspergillus* isolates (Sakko et al., 2014; Sakko et al., 2015). HICA is most active at pH 5.2. It is a biocompatible agent and it is being used at a 1500 mg daily dose as a nutritional supplement to increase muscle mass (Mero et al., 2010). HICA is also approved to be used as a flavouring substance in food manufacturing (Additives and Food, 2012).

Alpha-mangostin is a xanthone derivative from mangosteen (*Garcinia mangostana* Linn.) pericarp extracts and has been shown to possess biological activities including antioxidant, anti-tumoral, anti-inflammatory, and anti-allergy properties (Chomnawang et al., 2005; Obolskiy et al., 2009; Suksamrarn et al., 2003). Moreover, alpha-mangostin also has antimicrobial activity against a wide range of microorganisms (Pedraza-Chaverri et al., 2008; Suksamrarn et al., 2006). The planktonic MIC of alpha-mangostin to various bacteria and fungi ranges between 12,500-50,000 mg/L, 1,000-5,000 mg/L respectively (Chin et al., 2008). There are no studies on the anti-biofilm activities of the alpha-mangostin on bacterial or fungal biofilms.

In this study, the aim was to determine the activity of HICA and alpha-mangostin on preformed multi-species *in vitro* biofilms of *C. albicans*, *E. faecalis*, *L. rhamnosus*, and *S. gordonii* or *S. salivarius*. The hypothesis was that HICA and alpha-mangostin have anti-biofilm activity against multi-species biofilms of these species which are commonly found in endodontic infections.
3.3 Materials and methods

3.3.1 Study design

This study was performed to determine the activity of HICA and alpha-mangostin on pre-grown multi-species in vitro biofilms of species commonly found in endodontic infections. To mimic a complicated endodontic environment, multi-species biofilms of C. albicans, E. faecalis, L. rhamnosus, and S. gordonii or S. salivarius were grown on coverslips in 24-well plates (for visualisation) or 96-well plates (for metabolic assays) in RPMI for 48 hours (Figure 3.1). Inocula were prepared by mixing an equivalent of $1 \times 10^8$ cells/mL of each species of C. albicans, E. faecalis, L. rhamnosus, and S. gordonii or S. salivarius. Pre-grown biofilms were exposed to HICA and alpha-mangostin for 24 hours at pH 5.2. RPMI was used as a negative control and sodium hypochlorite and chlorhexidine were used as comparators. Metabolic (XTT) assays were used to determine biofilm metabolism and bright field and fluorescence microscopy were used to visualise and quantify biofilms. Differences between the effects of HICA and alpha-mangostin on metabolism and viability of exposed and control biofilms were compared statistically using a one-way ANOVA test. All biofilm experiments were done twice in triplicate. In total, 1,408 biofilms were used in the study.

Figure 3.1 Summary of the study design.
3.2.2 Media

RPMI-1640 (R0883, Sigma-Aldrich, Missouri, USA) with 2.0 g/L D-glucose was prepared with morpholinepropanesulfonic acid (MOPS) (Oxoid, Basingstoke, UK) and buffered to pH 7.4. The medium was filtered through a 0.22 µm membrane filter (Sigma-Aldrich, Missouri, USA) and stored at 4°C. The final concentration of RPMI-1640 used to prepare single species and multi-species biofilms were single strength (1x) and quadruple strength (4x), respectively.

3.3.3 Antimicrobial agents

D,L-HICA (TCI Europe, Zwijndrecht, Belgium) was prepared in a 5% (w/v) solution and alpha-mangostin (Sigma-Aldrich, Missouri, USA) was prepared in a 0.2% (w/v) solution separately in 2x RPMI medium. The pH was adjusted to 5.2 for both agents, then filtered through a 0.22 µm membrane filter. HICA was prepared fresh each time, while the stock solution of alpha-mangostin was stored at 4°C.

3.3.4 Culture conditions and formation of multi-species biofilms

The mixed species inoculum was prepared by growing planktonic cultures of four bacterial isolates: *E. faecalis, L. rhamnosus, S. gordonii* and *S. salivarius* on blood agar (Oxoid, Basingstoke, UK) and one isolate of *C. albicans* (for reference numbers see Figure 3.1). For bacteria, one colony was inoculated into 20 mL LB broth (Lennox, Massachusetts, USA) and grown overnight at 37°C in a shaking incubator (at 250 rpm maximum). The cells were harvested by centrifugation (4,500 rpm for 5 minutes), washed twice with sterile phosphate-buffered saline (PBS) (Sigma, Missouri, USA) and then re-suspended in RPMI-1640. The cell density was measured by using a spectrophotometer and adjusted to 0.1 OD at 600 nm, equivalent to 1.0x10^8 cells/mL. For *C. albicans*, the isolate was cultured on Sabouraud Dextrose (SAB) agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 48 hours before being used to check viability and purity (by culture and microscopy, respectively). One colony was then inoculated into 20 mL yeast peptone dextrose (YPD) broth (Melford, Ipswich, UK) and grown overnight at 37°C in the shaking incubator as detailed above. The cells were harvested by centrifugation (3,000 rpm for 5 minutes), washed twice with sterile PBS and then re-suspended in RPMI-1640.
To produce standardised biofilm inoculum, the cell density was measured by using a Neubauer haemocytometer and adjusted to $1.0 \times 10^6$ cells/mL.

The culture suspensions of the four microorganisms (three bacteria and one yeast) were mixed in equal proportions to create the multi-species biofilms inoculum. The suspensions were incubated in an anaerobic incubator (5% CO$_2$) at 37°C to develop into biofilms which were then grown for 48 hours before exposure to reagents.

### 3.3.5 Susceptibility testing

HICA was diluted from a freshly made stock 20,000 mg/L aqueous solution, pH 5.2, into RPMI broth while alpha-mangostin was diluted from a stock 64,000 mg/L aqueous solution pH 5.2, into RPMI broth. Chlorhexidine (CHX) digluconate (20,000 mg/L, Corsodyl$^\text{®}$ (Glaxo Smith Kline, Middlesex, UK) and 25,000 mg/L sodium hypochlorite (NaOCl, Davis Schottlander & Davis Ltd., Hertfordshire, UK) were used as positive controls; RPMI medium was used as a negative control to determine the maximum microbial growth.

Broth microdilution methods were used to determine the susceptibility of the planktonic *C. albicans* isolates (CLSI, 2008a) and *E. faecalis* (tested again to confirm findings in Chapter 2), *L. rhamnosus*, *S. gordonii*, *S. salivarius* isolates (CLSI, 2009) to HICA and alpha-mangostin. The minimum inhibitory concentration (MIC) was read spectrophotometrically after 24 hours of incubation. Microbial suspensions (20 μL) were added to broth (180 μL) in 96-well cell-culture microtiter plates (F96 MicroWell Plates, NUNC, ThermoFisher Scientific, Leicestershire, UK). Microbial growth was determined by a spectrophotometer Multiscan RC analyser (ThermoFisher Scientific) at 490 nm. The inhibitory concentration of HICA that prevented 50% and 90% of the bacterial or candidal growth (IC$_{50}$ and IC$_{90}$, respectively) was determined by changes in optical density. The tests were done with three replicate wells and repeated once. In addition, the microbiocidal activity of HICA and alpha-mangostin was tested after MICs were determined by streaking 20 μL of the test suspensions from representative wells onto agar and incubating for 48 hours; the results were shown as minimum bactericidal and fungicidal concentration (MBC/MFC).
3.3.6 Biofilm growth and treatment

For microscopic visualisation, multi-species biofilms were grown on Thermanox coverslips (Thermo Scientific Nunc, Illinois, USA) in RPMI-1640 medium at pH 7.4 in static 24-well plates (Corning CoStar, North Carolina, USA) for 48 hours at 37°C prior to exposure to 5% (w/v) DL-HICA, alpha-mangostin, RPMI-1640, NaOCl, and CHX for 24 hours at pH 5.2 at 37°C. For metabolic assays, 96-wells plate was used for direct analysis of the biofilms grown in situ without Thermanox cover slips.

3.3.7 Measuring the metabolic activity of multispecies biofilms with XTT reagent

Before analysis, biofilms were washed with sterile PBS. The XTT solution was prepared following a previously published study (Nieminen et al., 2014); briefly, saturated XTT in PBS was supplemented with menadione to a final concentration of 10 µM. Then 200 µL of XTT solution was added to each well in 96-well plates or 1 ml to each well in 24-well plates. The plates were incubated in the dark at 37°C incubator for 2 hours. After incubation, 100 µL of the XTT supernatant was transferred into separate wells in a fresh 96-wells plate and the colourimetric changes were measured spectrophotometrically (BMG Labtech, UK) at 490 nm.

3.3.8 Multi-species biofilm characterisation and quantification

The multi-species biofilm architecture was characterised by bright field and fluorescence microscopy. Pregrown biofilms were washed once with PBS before staining for 1 minute with LIVE/DEAD® BacLight™ viability kit (ThermoFisher Scientific, Leicestershire, UK), containing SYTO9 and propidium iodide (PI), according to the manufacturer’s instructions. Biofilms were transferred to microscope slides and examined under bright field and fluorescence microscopy (Nikon Eclipse 80i). To detect propidium iodide fluorescence, excitation was at 532 nm and emission at 635 nm (Tx Red) and to detect SYTO9 fluorescence, excitation was at 483 nm and emission at 503 nm (FITC). Images were processed using Image-Pro Plus 5.1 (Media Cybernetics, USA). The quantitative analysis was further conducted at 6,000-fold magnification on an Axioskop II microscope (Zeiss; Oberkochen, Germany). The number of cells was counted in ten randomised microscopic ocular
grid fields (0.0156 cm² grid area). Etbr filter was used for the visualisation of dead cells: BP 546/12, FT 580, and LP 590. FDA filter was used for the visualisation of vital cells: BP 450–490, FT 510, and LP 515. Moreover, the replicate sets of control and exposed biofilms were assessed for cell viability by culture of serial dilutions on agar plates and colony counting.

3.3.9 Statistical analysis

GraphPad Prism (version 7.03; La Jolla, CA, USA) was used for data analysis, to represent the data graphically and provide statistical analysis. One-way Analysis of Variance (ANOVA) was used to investigate significant differences between independent groups of data which followed normal Gaussian distribution (differences between HICA and alpha-mangostin treated biofilms compared to control conditions). To analyse the multiple comparisons of the data, a Bonferroni correction was applied to the $p$ value. Statistical tests were interpreted at the 5% significance level.

3.4 Results

3.4.1 Planktonic susceptibility testing

The effect of HICA and alpha-mangostin on two C. albicans, two E. faecalis, two L. rhamnosus, one S. gordonii, and one S. salivarius isolate grown planktonically is summarised in Table 3.1. Alpha-mangostin was shown to have antibacterial and antifungal activity: the IC₉₀ for bacterial isolates was 0.5 mg/L and it was bactericidal (MBC) at 2-4 mg/L and candidical (MFC) at 1,000 mg/L. Of all the tested isolates, E. faecalis were most susceptible to alpha-mangostin with an IC₉₀ of 2 mg/L while L. rhamnosus and S. gordonii isolates were less susceptible with an IC₉₀ of 8 mg/L. The bactericidal activity of alpha-mangostin against L. rhamnosus and S. salivarius was improved by reducing the pH from 7.4 to 5.2 (Table 3.2). HICA was bactericidal at 6,300-50,000 mg/L and candidical at 50,000 mg/L (this confirms the findings in chapter 2). E. faecalis isolates were most susceptible to HICA with an IC₉₀ of 1,600 mg/L whereas the L. rhamnosus and S. gordonii isolates had an IC₉₀ of 6,300 mg/L (Table 3.1).
Table 3.1 The activity of HICA and alpha-mangostin against *E. faecalis* and *C. albicans* isolates in acidic conditions.

<table>
<thead>
<tr>
<th>Organism and phenotype</th>
<th>Designation</th>
<th>Source</th>
<th>HICA (pH =5.2)</th>
<th>alpha-mangostin (pH =5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC₅₀ (mg/L)</td>
<td>IC₉₀ (mg/L)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>ATCC 10231</td>
<td>Man with broncho mycosis</td>
<td>50,000</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>D 17</td>
<td>Root canal</td>
<td>12,500</td>
<td>50,000</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>Urine</td>
<td>100</td>
<td>1,600</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>T 75359</td>
<td>Root canal</td>
<td>800</td>
<td>3,200</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>ATCC 53103</td>
<td>Faeces</td>
<td>3,200</td>
<td>6,300</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>T 75020</td>
<td>Root canal</td>
<td>3,200</td>
<td>6,300</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>T 75354</td>
<td>Root canal</td>
<td>6,300</td>
<td>6,300</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>ATCC 13419</td>
<td>Saliva</td>
<td>3,200</td>
<td>3,200</td>
</tr>
</tbody>
</table>

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; IC₅₀/₉₀, concentration that inhibits 50% and 90% of the growth, respectively.
Table 3.2 The activity of alpha-mangostin against *E. faecalis* and *C. albicans* isolates in neutral and acidic conditions.

<table>
<thead>
<tr>
<th>Organism and phenotype</th>
<th>Designation</th>
<th>Source</th>
<th>alpha-mangostin (pH =7.4)</th>
<th>alpha-mangostin (pH =5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC_{50} (mg/L)</td>
<td>IC_{90} (mg/L)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>ATCC 10231</td>
<td>Man with broncho-mycosis</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>D 17</td>
<td>Root canal</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>Urine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>T 75359</td>
<td>Root canal</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>ATCC 53103</td>
<td>Faeces</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>T 75020</td>
<td>Root canal</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>T 75354</td>
<td>Root canal</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>ATCC 13419</td>
<td>Saliva</td>
<td>0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; IC_{50/90}, concentration that inhibits 50% and 90% of the growth, respectively.

3.4.2 HICA and alpha-mangostin have a significant inhibitory effect on biofilm metabolic activity

Exposure to antimicrobial agents reduced the metabolic activity of all pre-grown biofilms significantly (p<0.0001) (Figure 3.2 and 3.3). Sodium hypochlorite inhibited the metabolic activity of single and multi-species biofilms by at least 98%. Both HICA and alpha-mangostin were most active against *L. rhamnosus* biofilms (metabolism inhibited by 98% and 99%, respectively). The inhibition of the metabolic activity of both alpha-mangostin and chlorhexidine on *E. faecalis*, *L. rhamnosus*, and *S. gordonii* was comparable. HICA and alpha-mangostin inhibited *S. gordonii* biofilms by 98%, they were comparable to sodium hypochlorite and chlorhexidine comparators. Alpha-mangostin had better activity against multi-species biofilms than HICA (93% inhibition vs 46% inhibition) (p<0.0001). The results of the clinical isolates were comparable to those of the reference isolates. The pH conditions had no effect on the anti-biofilm activity of alpha-mangostin (Figure 3.3).
Figure 3.2 Inhibition of metabolic activity in biofilms composed of a total of four clinical isolates (A) and reference isolates (B) after 24-hour exposure to the antimicrobials compared to the negative control measured by XTT assay. Results of two independent experiments done in triplicate are presented (**p<0.0001: one way- ANOVA with a Bonferroni correction).
Inhibition of metabolic activity in biofilms composed of a total of four clinical and reference isolates after 24-hour exposure to alpha-mangostin in acidic and neutral conditions compared to the negative control measured by XTT assay. Results of two independent experiments done in triplicate are presented (***p<0.0001, ns= not significant: one way-ANOVA with a Bonferroni correction).

3.4.3 Visualisation and quantification of biofilms

Biofilms were intact prior to exposure to antimicrobials reagents and some pseudo-hyphae were observed in the 48 hours fungal biofilms (Figure 3.4, panel a-b). In the control biofilms, 89.8% of candidal cells were viable. When assessed by LIVE/DEAD staining, HICA and alpha-mangostin exposure reduced the number of viable cells in single C. albicans biofilms. After exposure to HICA, 47.0% the candidal cells were viable, after exposure to alpha-mangostin, only 23.0% of candidal cells were viable. Moreover, both agents inhibited candidal hyphal formation (Figure 3.4, panel c-f). For bacterial isolates, the number of enterococcal cells that appeared to be viable after exposure to alpha-mangostin was comparable to that of HICA according to the results of staining. Alpha-mangostin reduced the number of
viable lactobacilli and streptococcal cells significantly more than HICA (p<0.001). Interestingly, lactobacilli were intact after exposure to HICA (Figure 3.6, panel d; Table 3.3).

In multi-species biofilms, the hyphal structure of candidal cells was observed after exposure to HICA and alpha-mangostin. However, all candidal cells appeared to be non-viable (Figures 3.8, panel c-f; Table 3.3). After exposure to HICA, the proportion of viable cells was reduced to 63.1%. After exposure to alpha-mangostin, only 7.0% of candidal cells stained viable (Figure 3.8, panel c-f; Table 3.3). Interestingly, 97.6% of the cells stained viable after exposure to CHX. Moreover, candidal cells appeared intact in both single and multi-species biofilms after CHX exposure (Figure 3.5-3.8; panel g-h).

**Table 3.3** Quantification of live and dead cells of control biofilms, and biofilms after exposure to HICA, alpha-mangostin (AM), and chlorhexidine (CHX). The quantification was done by LIVE/DEAD staining.

<table>
<thead>
<tr>
<th>Agents/ Biofilms</th>
<th>Candidal biofilms</th>
<th>Entercoccal biofilms</th>
<th>Lactobacillus biofilms</th>
<th>Streptococcal biofilms</th>
<th>Four-species biofilms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Live cells</td>
<td>% Dead cells</td>
<td>% Live cells</td>
<td>% Dead cells</td>
<td>% Live cells</td>
</tr>
<tr>
<td>Control</td>
<td>89.8</td>
<td>10.2</td>
<td>100.0</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>HICA</td>
<td>47</td>
<td>53</td>
<td>37.7</td>
<td>62.3</td>
<td>100.0</td>
</tr>
<tr>
<td>AM</td>
<td>23</td>
<td>77</td>
<td>35.0</td>
<td>65.0</td>
<td>0.1</td>
</tr>
<tr>
<td>CHX</td>
<td>4</td>
<td>96</td>
<td>94.8</td>
<td>5.2</td>
<td>88.3</td>
</tr>
</tbody>
</table>
Figure 3.4 Impact of HICA and alpha-mangostin on candidal cells in single species biofilm on the hyphal formation and morphology of *C. albicans* (D 17) by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (CHX; g-h).
Figure 3.5 Impact of HICA and alpha-mangostin exposure on enterococcal cells in single species biofilm on the morphology of *E. faecalis* (T 75379) by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (CHX; g-h).
Figure 3.6 Impact of HICA and alpha-mangostin on *Lactobacillus* cells in single species biofilm on the morphology of *L. rhamnosus* (T 75020) by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (CHX; g-h).
Figure 3.7 Impact of HICA and alpha-mangostin on streptococcal cells in single species biofilm on the cellular morphology of S. gordoni (T 75354) by bright field microscopy (left) and fluorescence microscopy using BacLight® (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (CHX; g-h).
Figure 3.8 Impact of HICA and alpha-mangostin on the morphology of candidal, enterococcal, lactobacilli, and streptococcal cells in multi-species biofilm by bright field microscopy (left) and fluorescence microscopy using BacLight™ (right): RPMI control (a-b), HICA (c-d), alpha-mangostin (e-f), and comparator (CHX; g-h).
After exposure to HICA, alpha-mangostin, or CHX, the number of CFU/mL was reduced by 3-Log and the inhibitory activity of alpha-mangostin and CHX was comparable (Figure 3.9).

**Figure 3.9** Quantification of viable cells (CFU/mL) of control biofilms, and biofilms after exposure to HICA, alpha-mangostin (AM), and chlorhexidine (CHX) by culture. The means were presented in triplicate (**p<0.0001: one way- ANOVA with a Bonferroni correction).
3.5 Discussion

HICA and alpha-mangostin were found to be bactericidal and fungicidal against polymicrobial biofilms in our four-species model. Alpha-mangostin was cidal against all planktonically grown isolates at 1,000 mg/L and reduced the metabolic activity of the mixed-species biofilms by >80% at 2,000 mg/L. On the other hand, HICA was cidal against all planktonically grown isolates and reduced the metabolic activity of the mixed-species biofilms by >40% at 50,000 mg/L. Interestingly, the effective anti-biofilm concentration of these agents was not significantly higher than for planktonic microbes as commonly reported for current antimicrobials (Cerca et al., 2005; Davies, 2003; Stewart and Costerton, 2001). For example, fluconazole MIC for C. albicans typically increases by 1000x when it transforms into biofilm growth (Casalinuovo et al., 2004).

The impact of antimicrobials on the biofilms appeared to be more striking if measured by colony counting or by viability staining than by the metabolic assay. This may be partially due to the induction of cellular processes under stress caused by the antimicrobial exposure, and the cells being relatively more active metabolically. On the other hand, culture is known to overestimate the number of cells which produce hyphae (Falsetta et al., 2014). As all antimicrobial agents tested in this study resulted in reduction in the production of hyphae, this would exaggerate their impact on cell viability.

Fluorescence microscopy with viability staining was used to visualise the impact of the antimicrobials on the biofilm viability. Interestingly, L. rhamnosus biofilms were intact and viable after exposed to HICA. This finding can be explained by the ability of lactobacilli to metabolise HICA by using D-hydroxyisocaproate dehydrogenase (HicDH) (Bernard et al., 1991). Also, after exposure to CHX, all biofilms stained green suggesting they were viable, although parallel biofilms assayed with XTT and culture showed no metabolic activity or growth. However, notable damage to the biofilms could be seen and it is possible that positive signal reflected non-specific binding to the damage cell walls. In these biofilms, viability staining may underestimate the viable cell because the impact of CHX on the bacterial or fungal cell wall. CHX damages the cell walls of the bacteria (Cheung et al., 2012) and fungi (Suci and Tyler, 2002), and leads to leakage of the cellular...
contents. If these becoming wash away during staining process, PI would give the false negative signal.

There are some limitations of this study. Firstly, only four isolates of microorganisms were used in the model. In clinical situations, polymicrobial flora with an average of four to seven intra-canal species have been recovered from the necrotic pulpal tissues in primary root canal infections; these microbes were mostly Gram-negative obligatory anaerobic bacteria (Rôças et al., 2004b; Kayaoglu and Ørstavik, 2004; Rôças et al., 2004c; Schleifer and Kilpper-Bälz, 1984; Gajan et al., 2009; Siqueira and Rôças, 2005; Schäfer and Bossmann, 2001). However, this study was focused on the treatment of secondary or persistent infections which Gram-positive facultative anaerobes, especially \textit{E. faecalis}, were predominant (Tronstad and Sunde, 2003; Kayaoglu and Ørstavik, 2004; Gajan et al., 2009; Schäfer and Bossmann, 2001). Secondly, the antimicrobial activity of alpha-mangostin was not tested in the presence of potential inhibitors such as hydroxyapatite, dentine, albumin and other serum proteins present in infected root canals. This topic requires further study in order to evaluate the potential of the agent to be used in endodontics. Finally, this study is performed \textit{in vitro}. To mimic the environments in clinical situations, the antimicrobial activity of HICA and alpha-mangostin against multi-species biofilm in an \textit{ex vivo} tooth model is required.
3.6 Conclusion

HICA and alpha-mangostin were found to be bactericidal and fungicidal against polymicrobial biofilms in our four-species model. The impact of HICA and alpha-mangostin on *C. albicans*, *E. faecalis*, *L. rhamnosus*, and *S. gordonii* or *S. salivarius* and the mixed biofilms composed of these species is shown in this study. Both HICA and alpha-mangostin inhibited the metabolic activity of bacterial-fungal biofilms effectively. The anti-biofilm activity of alpha-mangostin is comparable to that of highly active but toxic CHX. A higher concentration of HICA is required to be as active as alpha-mangostin. These two novel antimicrobial agents are consistent with potential use as a topical agent of superficial fungal, bacterial or multi-species microflora infections, they could also be suitable for the treatment of infected body cavities due to their good biocompatibility and safety profile. Further *ex vivo* studies using infected root canal models and clinical trials are required before the clinical use of HICA and alpha-mangostin as the inter-appointment intra-canal medicaments.
3.7 References


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Chapter 4
Efficacy of HICA and alpha-mangostin against multi-species biofilm in an ex vivo tooth model
4.1 Abstract

**Background:** Multi-species biofilms are commonly associated with diseases including root canal infections. In endodontic treatment, biofilm removal is accomplished by a chemo-mechanical process. Inter-appointment medication is used to further improve the antimicrobial effect. Currently, calcium hydroxide paste is the most widely used medicament in endodontics, ostensibly due to its high pH and antimicrobial properties. However, there are conflicting results regarding this antimicrobial activity. The potential tissue toxicity and allergic reactions of the medicament are of concern. Effective disinfection of dentine during root canal treatment remains a major challenge with existing medicaments and there is an argument for the development of novel antimicrobial agents with a different mode of action. HICA (D,L-2-hydroxyisocaproic acid) is a protein fermentation product, which has antimicrobial activity against bacteria and fungi. It also has anti-inflammatory activity. Alpha-mangosin \( (1,3,6\text{-trihydroxy}-7\text{-methoxy}-2,8\text{-bis(3-methylbut-2-enyl)xanthen-9-one}) \) is a xanthone-derivative which possesses antimicrobial properties against a wide range of microorganisms. Creating complex biofilm models that represent the polymicrobial environment of root canal infections is important in the development of new antimicrobial agents. The ability to undertake compositional analysis is required to accurately understand the nature of each species in a biofilm. Moreover, it is possible to determine individual cell viability by molecular approaches. PMA-qPCR (quantitative PCR with propidium monoazide) methods can be used to show the viability of microbes as well as analyse the composition within polymicrobial root canal biofilms before and after treatment.

**Aims:** To develop a defined multi-species root canal biofilm model ex vivo, and to perform viable compositional analysis following HICA, alpha-mangostin, Calcicur®, and Odontopaste® exposure.

**Methodology:** Time-kill assays were conducted using HICA, alpha-mangostin, Calcicur®, Odontopaste®, and saline solution on the planktonic cultures of C. albicans, E. faecalis, L. rhamnosus, and S. gordonii. Multi-species biofilms were incubated within the root canals of 1.4 mm diameter inside root blocks \((n = 100)\) for 21 days. Canals \((n = 20/\text{group})\) were then exposed to 40% (w/v) HICA paste, 1% (w/v) alpha-mangostin solution, Calcicur®, and Odontopaste® paste or 0.9% (w/v) saline solution for 7 days. Samples taken from the inner (first 0.1 mm) and deeper
(second 0.1 mm) dentine, and residual roots were cultured in broth for 24 hours. Cell growth was detected by spectrophotometry (optical density, OD) and confirmed by culture on agar. The other set of samples were sonicated then exposed with 50 μM PMA before DNA was extracted using the QIAamp DNA mini kit. Real-time quantitative PCR (qPCR) was performed to determine the biofilm composition as well as the number of live and total cells remaining in the biofilm following each treatment. The OD data were analysed with Kruskal–Wallis and Friedman with Wilcoxon signed-rank test between and within groups, respectively, and agar culture data with Pearson chi-square with Mann–Whitney and Cochran with McNemar tests, respectively (P<0.05).

**Results:** HICA and Calcicur® killed all planktonic organisms within 24 hours, while alpha-mangostin killed the organisms within 72 hours. However, Odontopaste® was a slow-killing agent: 10 cells of planktonic organisms survived after exposure to the agent for 7 days. **Conclusions:** Using this model and methodology, cell viability was reduced and population dynamics were altered when biofilms grown in root canals were exposed to these potentially new medicaments: both HICA and alpha-mangostin showed superior activity to conventional reagents. Therefore, these agents could have potential to be utilised as inter-appointment medicaments in root canal treatment.

**4.2 Introduction**

Microorganisms have long been identified as the main cause of the development of infection that involves the inner root canal space of a tooth, within the dentine itself and on the outer root walls (Pinheiro et al., 2003). Reinfection and continued inflammation of the tissue beyond the root apex may occur from proliferation of microorganisms residing in the anatomic complexities of the root canal system and dentinal tubules (Siqueira et al., 2002). In root canals, microorganisms commonly exist in the form of a biofilm, a complex structure adhering to surfaces regularly in contact with water and consisting of bacterial colonies and usually other microorganisms such as yeast, fungal, and protozoan communities encased in a mucilaginous protective coating, and thus difficult to eradicate and often resistant to antimicrobial agents (Donlan and Costerton, 2002). Biofilms in root canals are less diverse compared with oral biofilms (Jhajharia et al., 2015). Infections in root canals alter the nutritional and environmental status, which
is anaerobic, and nutritional levels are depleted (Narayanan and Vaishnavi, 2010). These changes offer a harsh ecological condition for the surviving microorganisms. Moreover, persistent microorganisms can invade the anatomical complexities and apical portions of the canals (Frank and Barbour, 2006; Hornef et al., 2002).

Endodontic infections are typically of polymicrobial etiology (Deng et al., 2009; Rôças et al., 2008). In cases of failed endodontic treatment and canals with persistent infections, Enterococcus faecalis and Candida albicans have been identified (Gopikrishna et al., 2006). The complex structure of the biofilm allows some degree of cooperation to develop between the species populations (Romano and Kolter, 2005; Seneviratne et al., 2008).

Antimicrobial irrigating solutions and medicaments are used to eradicate microorganisms from root canal systems. A broad antimicrobial spectrum and high efficacy against microorganisms in biofilms are the ideal properties of a root canal medicament. Moreover, the medicament should be biocompatible and non-caustic to periodontal tissues (Kennedy and Hussey, 2007; Schäfer, 2007). Currently calcium hydroxide (Ca(OH)$_2$) paste is the most widely used inter-appointment medication in endodontics (Haapasalo and Shen, 2010). The mode of action of Ca(OH)$_2$ is based on its alkalinity, but there are conflicting results of its antimicrobial activity (Peters et al., 2002; Vera et al., 2012). For example, Gram-positive bacteria, especially E. faecalis, have been reported to survive in an alkaline environment (Byström et al., 1985; Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990; Safavi et al., 1990), and are unaffected by Ca(OH)$_2$ as a canal medicament. Moreover, potential tissue toxicity and allergic reactions are of concern (Guleri et al., 2012; Pasternak and Williamson, 2012; Gomes et al., 2013). Therefore, there is a need for agents which possess both antimicrobial activity and exert minimal tissue-irritating effects.

D,L-2-hydroxyisocaproic acid (HICA) has the ability to inhibit the growth of planktonically grown pathogenic fungi including Candida and Aspergillus (Sakko et al., 2014; Sakko et al., 2015). HICA also has bactericidal activity against Gram-positive and Gram-negative bacteria (Sakko et al., 2012; Sakko et al., 2014). Considering the antimicrobial activity of HICA, it could be a good choice for mixed bacterial-fungal infections (Nieminen et al., 2014).

Alpha-mangostin is a xanthone derivative from mangosteen extracts that have been reported to exert potent antimicrobial activity against several Gram-positive and
Gram-negative bacteria including *Staphylococcus aureus* (both normal and methicillin-resistant), *Staphylococcus epidermidis, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhimurium, Enterococcus* species, *Mycobacterium tuberculosis* and *Propionibacterium acnes* (Chomnawang et al., 2005; Obolskiy et al., 2009; Suksamrarn et al., 2003; Torrungruang et al., 2013). Moreover, alpha-mangostin has been shown to have antifungal properties against various fungal species including *C. albicans* (Gopalakrishnan et al., 1997; Kaomongkolgit et al., 2009). However, there are no studies on the anti-biofilm activities of alpha-mangostin on bacterial or fungal biofilms.

For the root canal medicaments, a time-kill test is the most relevant method for determining bactericidal or fungicidal effects as it reflects the clinical scenario. It is an effective tool for obtaining information about the dynamic interaction between the antimicrobial agents and the microbial isolates (Pfaller et. al., 2004). The time-kill test reveals the time required for maximal effect for each medicament and isolate. This information can be used to decide the duration and concentration of medicaments in the *ex vivo* root canal model.

The complex composition of multi-species microbial communities hinders the ability of conventional microbiology to sensitively quantify the microorganisms present. Therefore, alternative molecular approaches are warranted when assessing the viability of biofilms (Sherry et al., 2016). Molecular methods are more sensitive than culture/microscopy techniques for determining viability and for enumeration and identification of species. Previous studies carried out on various oral bacteria biofilms showed that the viable and dead cells from multi-species biofilms can be differentiated by a live-dead PCR method (Sanchez et al., 2013; Sánchez et al., 2014; Álvarez et al., 2013). Propidium monoazide (PMA) can be used as a viability indicator with real-time polymerase chain reaction (qPCR). When exposed to light, PMA covalently binds to DNA bases every 4–5 nucleotides and forms a carbon–nitrogen bond. This bond inhibits further amplification of extracellular DNA or DNA contained in dead cells; therefore, the amplification of DNA by PCR is allowed from only the viable cells present in the sample (Nocker et al., 2006). The PMA-qPCR method can be used to assess the effect of medicaments or cleaning regimens on complex multi-species biofilm models (Sherry et al., 2016).
The complexity of the environment in the human root canal system requires further testing in clinically relevant conditions. The hypothesis is that HICA and alpha-mangostin could be potentially novel agents for difficult-to-treat root canal infections. The aims of the present study were to develop a multi-species biofilm model that was representative of a root canal environment within dentinal tubules and to devise a rapid and sensitive method to quantify the viable composition of biofilms challenged with HICA and alpha-mangostin in comparison to conventional root canal medicaments.

### 4.3 Materials and methods

#### 4.3.1 Study design

Extracted human single-root teeth (n=100) were collected and autoclaved. The roots were cut to a standard length, then the root canal lumen size was standardised and rinsed with sterile saline. The outside surfaces of the root samples were coated with fingernail polish (Superdrug, Surrey, UK), and the apical foramen were sealed with Tenatex pink toughened modelling wax (Dental Sky Ltd., Kent, UK) and super glue (Henkel Ltd., Herts, UK) to avoid contamination of the root canals. The root samples were embedded into 24-well plates with Silagum-Putty dental silicone impression material (DMG Chemisch-Pharmazeutische, Hamburg, Germany). Then, they were cultured with the four isolates for 21 days, and randomly assigned to five medicament groups (HICA, alpha-mangostin, Calcicur®, Odontopaste® and saline solution). After 7 days exposure, samples taken from the inner (first 0.1 mm) and deeper (second 0.1 mm) dentine, and the residual roots were cultured in broth for 24 hours. Cell growth was detected by spectrophotometry (optical density, OD) and was confirmed by culture on agar (Figure 4.1). For the molecular method, PMA (50 μM) was added to each sample and DNA was extracted; then biofilm composition and viability were assessed by real-time quantitative PCR to determine the effectiveness of the medicaments. The ex vivo experiment was done in triplicate. Time-kill assay results were used to decide the exposure time.
Figure 4.1 Root canal infection model; root canals of human dental root blocks were infected with a multi-species biofilm and then exposed to HICA paste, alpha-mangostin solution, Calcicur® paste, Odontopaste® paste or saline. Spectrophotometric analyses of the resulting cultures in broth and viability testing on agar were performed to confirm growth. The biofilm composition and viability were assessed by real-time quantitative PCR. The results were presented as cell growth inhibition, prevention of cell growth and bactericidal/fungicidal activity.

4.3.2 In vitro time kill assay

Cultures of C. albicans D 17, E. faecalis T 75359, L. rhamnosus T 75020, and S. gordonii T 75354 grown overnight in brain heart infusion (BHI, Sigma-Aldrich, Dorset, UK) broth at 37°C with shaking at 250 rpm were centrifuged at 2500 rpm for 10 minutes and washed with phosphate buffer saline (PBS) and finally suspended in BHI medium. The medicaments and a control (sterile water) were incubated with an adjusted amount of each species equal to 1.5x10⁸ CFU/mL in BHI medium in 96-well plates. Cells were harvested at 0, 2, 4, 6, 12, 24, 36, 48 hours. The cells were also harvested every day until day seven after inoculation. Two-fold serial dilutions were prepared and 10 μL of each dilution was spread on BHI agar plates. After overnight
incubation at 37°C, the number of colony forming units (CFUs) was counted. These procedures were done twice in three biological replicates.

4.3.3 Tooth collection, storage, and sterilisation

One hundred permanent human single-root teeth were collected and were stored in 10% (v/v) formalin at room temperature (Attam et al., 2009). The teeth were extracted as part of orthodontic treatment and the collection of the extracted teeth was approved by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University, Thailand. Written consent for collection was obtained from the patient or their guardian. The approval of the University of Manchester Research Ethics Committee was obtained for this study (Ref 16445). The inclusion criteria stated that only single root canal teeth with no root curvatures or fractures were collected, and no oval root canal-shaped teeth were selected. All teeth were autoclaved for 20 minutes at 15 psi; this was to ensure that all samples were free from infection and that viable DNA was eliminated. The teeth were then stored in 0.1% (v/v) thymol solution. The vessels containing the teeth were sealed tightly and then packed in foam boxes before transportation. After transportation to the Education & Research Centre, Wythenshawe Hospital, Manchester the external root surfaces were debrided of bone, calculus, and soft tissue using a No. 15 Surgical Scalpel Blade (Swann-Morton, Sheffield, UK). Teeth were cut with a diamond disc (Cookson Dental, Birmingham, UK) into six-mm-long and at least four-mm-wide standard root blocks. Root canals were enlarged with Ash Steel Bur No. 4 (Henry Schein Dental, Cardiff, UK) drills under water-cooling to a 1.4 mm diameter to standardise the samples.

Smear layers were removed from the canal walls in an ultrasonic bath with 17% (w/v) EDTA and 0.5% (v/v) NaOCl, 5 minutes for each. The blocks were autoclaved at 121°C for 30 minutes in 3 mL BHI broth. They were then sonicated in fresh broth in an ultrasonic bath for 15 minutes and finally cultured at 37°C and 5% CO₂ for 24 hours to confirm sterility. The growth conditions at 37 °C in CO₂ for 24 hours were constant in every phase of the study. The root canals were dried with sterile paper points (Orbis, Pontault-Combault, France), and the outer surface of the root blocks were covered with nail varnish (Rimmel London Ltd., London, UK) to avoid dehydration during the experiment. The apical-ends of root blocks were sealed with Tenatex pink toughened modelling wax. The blocks were stabilised in a vertical
orientation with the root canal opening at the top and embedded firmly into putty-type silicone impression material (Henry Schein Dental, Cardiff, UK) in the wells of sterile 24-well plates (Costar, Corning Incorporated, Corning, NY, USA) in a random order (Figure 4.2). By embedding in this way no liquid escaped through the bottom of the root canal. For the incubations, the plates were covered with sterile lids to ensure sterility throughout incubation.

Figure 4.2 Schematic representation of the Root canal infection model; the root block was embedded in the 24-well plates.

4.3.4 Multi-species biofilm formation in a tooth model

The root canals were filled with the multi-species suspensions containing each of $1.5 \times 10^8$ CFU/mL C. albicans, E. faecalis, L. rhamnosus, and S. gordonii in BHI using a 26-gauge needle (Sigma-Aldrich, Dorset, UK). Fresh cell suspension was delivered every second day, and pure broth (BHI) was delivered the days in between. All of the root blocks were cultured for 21 days at 37 °C in order to allow biofilm formation and maturation within the canals.

4.3.5 Medication

Antimicrobial activity of the agents was tested in the root canals over a 7-day period. The inoculated root blocks were randomly assigned to five groups (n = 20). The root canals were irrigated with 10 mL of sterile saline and vacuum suction was used during irrigation. The root canals were then dried with paper points. HICA was
prepared in deionised water at 400,000 mg/L (40% w/v) and the pH was adjusted to 5.2 (Hietala et al. 1979). Alpha-mangostin was prepared in deionised water at 10,000 mg/L (1% w/v) and the pH was adjusted to 5.2. Two commercial root canal medicaments were used as antimicrobial comparators. The first agent was Calcicur® (VOCO GmbH, Cuxhaven, Germany) which is a calcium hydroxide-based paste (containing 45% (w/v) calcium hydroxide). The second agent was Odontopaste® (Australian Dental Manufacturing, Kenmore Hills, Qld, Australia) which is a steroid/antibiotic paste containing 5% (w/v) clindamycin hydrochloride and 1% (w/v) triamcinolone acetonide. Sterile saline (0.9% w/v) was used as an inactive negative control for cell growth. All agents were delivered once into root canals, which were then closed with RelyX Unicem (3M Espe; Dental Products, Seefeld, Germany) to seal the top of the root canal to avoid evaporation.

4.3.6 Sampling

After 7 days of medication, the root canal surfaces were irrigated with 10 mL of sterile saline and the canals were dried with paper points. The samples of inner and deeper dentine (first 0.1 mm and second 0.1 mm, respectively) were collected separately with a sterile Ash Steel Bur No. 5 (diameter 1.6 mm) and an Ash Steel Bur No. 6 (diameter 1.8 mm) drills, respectively (Figure 4.3). The drills were moved gently down and up three times through the root canal with low rotational frequency (<1000 r/min). Dentine powders on the surface of the drill were delivered and mixed into 2 mL BHI in Eppendorf tubes. For successful transport of dentine samples into the broth, the drills were checked to ensure that they were clean to the naked eye. The broth containing dentine was vortexed. The residual roots were placed into separate tubes containing the same amount of broth and vortexed carefully before incubation. All samples were cultured for 24 hours at 37°C with shaking at 250 rpm.

After incubation, 200 µL of each suspension were delivered into 96-well microtiter plates (NUNC; ThermoFisher Scientific). The changes in optical density (OD450; Multiskan RC, version 6.0; Thermo Fisher Scientific) of the broth, compared to sterile control broth, were detected as cell growth. For viability testing, 20 µL of all test suspensions were cultured on Columbia agar supplemented with defibrinated horse blood (Oxoid Limited, Hampshire, UK) and colonies were counted after incubation at 37°C for 24 hours. Based on spectrophotometric analyses, the results were reported as cell growth inhibition (%) and as prevention of cell growth (no
growth %) with respect to the presence and absence of cell growth of the dentine or residual root suspensions in broth. Bactericidal and fungicidal activities were reported as percentage of negative cultures on agar. Culture purity was checked before and after the experiment using a light microscope.

Figure 4.3 Schematic representation of the circumferential sampling of the dentine. Root canals initially standardised with Ash Steel Bur No. 4 drill were enlarged with Ash Steel Bur No. 5 (inner dentine sample) and Ash Steel Bur No. 6 (deeper dentine sample) drills, and the dentin shavings were collected for analysis of cell growth.

4.3.7 Biofilm viability analysis by qPCR

4.3.7.1 Differentiation of total and live cells within biofilms

Quantitative analysis was performed to assess the effectiveness of each medicament against the multi-species root canal biofilms. In order to enumerate the definitive and relative composition of the biofilms, the viability of the treated biofilms was measured and compared using PMA-qPCR methods. Dentine samples were prepared by adding 50 μM PMA (final concentration) to what volume of material and the tubes were sonicated and then incubated in the dark for 10 minutes on ice to allow uptake of the dye. A 650 Watt halogen light was used to expose the samples for 5 minutes to facilitate covalent binding of PMA to the DNA in the samples. Then,
DNA was extracted using the QIAamp DNA mini kit, as per manufacturer’s instructions (Qiagen, Crawley, UK). In order to determine total biomass, replicate samples without PMA were also included as controls. A POLARstar Omega plate reader (BMG LABTECH Ltd., Bucks, UK) was used to check the quality of extracted DNA: samples with a 260/280 nm ratio of 1.8 to 2.2 were deemed to be of high quality and were used in subsequent PMA-qPCR procedures (Sherry et al., 2016).

### 4.3.7.2 Quantitative analysis of biofilm composition

The samples containing DNA, from live cells only (with PMA), and samples representing total DNA, from live and dead cells (without PMA) were analysed by real-time qPCR using a technique based on a previously published method (Sherry et al., 2016). Briefly, 1 μL of extracted DNA was added to a reaction mix containing Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies LDA UK Limited, Cheshire, UK) and 0.4 μM forward and reverse primers for each bacterial/fungal species. The primers used in the study were previously published (Table 5.1). The thermal profile used for amplification consisted of an initial denaturation of 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. Three independent biological replicates for each exposure including controls (plus/minus PMA), were amplified in triplicate using an MxPro Quantitative PCR machine and MxPro 3005P software (Stratagene, Amsterdam, Netherlands).
### Table 4.1 Bacterial and fungal primers for real time PMA-qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>References</th>
</tr>
</thead>
</table>
| **C. albicans** | F—GGGTGTGCATTGAAAGACGGTA  
R—TTGAAGATATACGTGGTGACGGTA | (Sherry et al., 2016)              |
| **E. faecalis** | F—CCGAGTTGATTGCACCTACATTGG  
R—CTCTTATGCAATCGCCGCTAAAC | (Sedgley et al., 2004)             |
| **L. rhamnosus**| F—CGG CTG GAC CTC CTT T  
R—GCT TGA GGG TAA TCC CCT CAA | (Haarman and Knol, 2006)          |
| **S. gordonii** | F—CGGATGATGCTAATCAAGTGACC  
R—GTTAGCTGTGGATTGCTTGGCC | (Álvarez et al., 2013)             |
| 16S             | F—CGCTAGTAATCGTGGATCACAGATGTG  
R—TGTGACGGGGCATGTGTA | (Suzuki et al., 2004a;  
Sherry et al., 2016)            |
| 18S             | F—CTCGTATGTTGACCTTGGGC  
R—GTCCTGCTTGGAAACACTCTA | (Sherry et al., 2016;  
Rajendran et al., 2015)         |

Melting curve analysis was performed for all samples to ensure a single peak, which was indicative of primer specificity. Primer efficiency was also confirmed to be consistent among all the primer sets used, by amplifying specific templates at six 10-fold dilutions. Samples were quantified to calculate the colony forming equivalent (CFE) based upon a standard curve methodology of bacterial/fungal colony forming units ranging from $1 \times 10^3$ to $10^8$ CFU/mL. Three independent biological replicates for each species at each of the concentrations were performed in triplicate and amplified as detailed above. Standard curves comparing the log (CFU/mL) to the Ct values were generated for each species with or without exposure to PMA and including total bacteria and total yeast. Linear equations representing the slopes of each comparison were generated (Table 5.2; Figure 5.2) so that the total (without PMA) and live (with PMA) concentration of cells in biofilms exposed to medicaments could be calculated from the Ct Value derived by PMA-qPCR. The concentration of dead cells was calculated by subtracting the live from total cell concentrations. The percentage of dead cells was derived from total cell concentrations, and the percentage of each species was derived from the total viable cells.
Table 4.2 Linear equations for generating the standard curves by quantitative real-time PCR of total cells (samples without exposure to PMA) and only viable cells (samples with exposure to PMA).

<table>
<thead>
<tr>
<th>Species</th>
<th>With PMA</th>
<th>Without PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>$y = -2.790x + 38.76$ \ $R^2 = 0.916$</td>
<td>$y = -2.546x + 36.43$ \ $R^2 = 0.928$</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>$y = -2.290x + 35.06$ \ $R^2 = 0.591$</td>
<td>$y = -2.033x + 34.75$ \ $R^2 = 0.580$</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>$y = -2.053x + 32.31$ \ $R^2 = 0.899$</td>
<td>$y = -2.454x + 33.10$ \ $R^2 = 0.846$</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>$y = -2.094x + 25.25$ \ $R^2 = 0.788$</td>
<td>$y = -2.535x + 32.86$ \ $R^2 = 0.772$</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>$y = -2.245x + 34.41$ \ $R^2 = 0.997$</td>
<td>$y = -2.083x + 35.54$ \ $R^2 = 0.944$</td>
</tr>
<tr>
<td>Total yeast</td>
<td>$y = -2.53x + 38.62$ \ $R^2 = 0.895$</td>
<td>$y = -2.235x + 35.54$ \ $R^2 = 0.795$</td>
</tr>
</tbody>
</table>

**Figure 4.4** The standard curves by quantitative real-time PCR of total cells (samples without exposure to PMA) and only viable cells (samples with exposure to PMA) for each isolate.
4.3.8 Statistical analysis

The statistical analysis was performed with GraphPad Prism Version 7.03 (GraphPad Software Inc., CA, USA) and IBM SPSS Statistics Version 20 (SPSS Inc, Chicago, IL, USA). One-way Analysis of Variance (ANOVA) and t tests were used to investigate significant differences between independent groups of data which followed normal Gaussian distribution. To analyse the multiple comparisons of the data, a Bonferroni correction was applied to the p value. The Mann-Whitney U-test or the Kruskal-Wallis test with a Dunn’s post-test was used for non-parametric data to assess differences between independent sample groups. Statistical tests were interpreted at the 5% significance level.

4.4 Results

4.4.1 Time-kill assays

The results of time-kill assays revealed that all medicaments inhibited the growth of planktonic microorganisms to different extents. The killing efficacy of the tested medicaments was time-dependent (Figure 4.4, 4.5). HICA and Calcicur® inhibited completely the growth of planktonic organisms within 24 hours, while alpha-mangostin killed all planktonic organisms within 72 hours. Use of Odontopaste® did not achieve 100% killing even after seven days (Figure 4.5). In the control groups, growth increased within the first six hours, then it declined gradually by 48 hours (Figure 4.4). At 24 hours, the reduction in growth by HICA, alpha-mangostin, and Calcicur® of C. albicans was 8-Log compared to the control. In comparison, the reduction by Odontopaste® was just 3-Log compared to control. HICA and Calcicur® showed 8-Log reduction for bacterial isolates, while there was only 1-Log reduction by alpha-mangostin and Odontopaste® (Figure 4.4). At 7 days, the reduction in growth by HICA, alpha-mangostin, and Calcicur® of C. albicans and the bacterial isolates was 5 to 7-Log compared to control. In contrast, there was only a 3 to 5-Log reduction by Odontopaste® (Figure 4.5).
Figure 4.5 48 hours growth of C. albicans, E. faecalis, L. rhamnosus, and S. gordonii in quantitative in vitro assays to measure the antimicrobial activity of 40% (w/v) HICA paste, 1% (w/v) alpha-mangostin solution, Calcinur® paste, and Odontopaste® paste. Microorganisms were grown in BHI media for up to 48 hours. Incubations were performed in replicate sets of Sterilin™ 5 mL polystyrene bijou containers containing 1 mL microbial suspensions combined with 1 mL of each medicament. Viability was determined at the indicated time points by serial dilution plating. The time-kill curves presented here are time points in 0, 2, 4, 6, 12, 24, 36, 48 hours. Results of two independent experiments done in triplicate are presented.
4.4.2 Ex vivo tooth model: spectrophotometric analyses for bacterial/fungal growth inhibition.

The percentage of cell growth inhibition was calculated by the decrease in OD due to each medicament compared to the OD of the saline control. HICA and alpha-mangostin significantly inhibited the cell growth in all sampling depths ($p<0.0001$) (Figure 4.6). Odontopaste® was the most active agent in inner dentine and residual roots: it inhibited the cell growth by 54% and 82%, respectively. The antimicrobial activity of Calcicur® was inferior to other medicaments at all sampling depths: it inhibited only 32% of the growth in the deeper dentine samples and it was nearly inactive in the residual roots. Differences in the killing activity of each medicament
between dentine depths were significant (p<0.0001). The activity of alpha-mangostin and Calcicur® was better in the deeper dentin than inner dentine or residual roots. However, the activity of HICA and Odontopaste® was better in the residual roots than in the inner and deeper dentin (Figure 4.6).

Figure 4.7 OD of samples (A), and the Mean cell growth inhibition (%) compared to negative control (B) in the inner (first 0.1 mm) and deeper (second 0.1 mm) dentine and in the residual roots with standard error of mean. Samples measured spectrophotometrically 24 hours after incubation. Prior to sampling, the roots were treated for 7 days of medication with 40% (w/v) HICA paste, 1% (w/v) alpha-mangostin solution, Calcicur® paste (positive control), Odontopaste® paste (positive control) or 0.9% (w/v) saline solution (negative control), in equal to three each. Statistical analysis of treatments was compared to their respective untreated controls (*p < 0.05, **p < 0.01, ***p < 0.001).
4.4.3 *Ex vivo* tooth model: viability control for bactericidal/fungicidal activity.

The relative efficacy of biofilm clearance was calculated by comparing the number of roots with bacterial/fungal growth in roots exposed to medicaments relative to those with microbial growth in saline control roots. Microbial growth was detected in 100% of the roots (20 roots) in the saline control group. There were significantly more roots with microbial cell growth detected after exposure to Calcicur® and Odontopaste® compared to HICA and alpha-mangostin in all dentine depths (p<0.0001) (Figure 4.7).

In the inner dentine, candidal cells were detected in 2-3% of roots exposed to HICA or alpha-mangostin, whereas bacteria were detected 41-43% of the roots (Figure 4.7). In inner dentine and residual roots, HICA and alpha-mangostin were almost equally effective at inhibiting growth of candidal and bacterial cells (p=0.8079) but alpha-mangostin was better than HICA in deeper dentine (p<0.0001) (Figure 4.7). All medicaments were least effective in residual roots (p<0.0001) (Figure 4.7).
**Figure 4.8** Relative number of roots with detectable cell growth (%) by culture technique is presented in different dentine depths: (A) inner dentin, (B) deeper dentine, and (C) residual roots. Samples from each dentine depth were cultured after 7 days exposure to 40% (w/v) HICA paste, 1% (w/v) alpha-mangostin solution, Calcicur® paste, Odontopaste® paste and 0.9% (w/v) saline (n = 20/group). Statistical analysis of treatments was compared to their respective untreated controls (*p < 0.05, **p < 0.01, ***p < 0.001).
4.4.4 Quantitative analysis of a multi-species root canal biofilm model

Exposure of multi-species biofilms to various root canal medicaments was initially quantified by CFU for total bacteria and yeasts (Figure 4.8). Previous growth analysis demonstrate that all medicaments significantly reduced CFUs in inner and deeper dentine and in residual roots. In this chapter, the analysis by PMA-qPCR showed the composition of different microbes within the biofilms (Figure 4.9).

In inner dentine samples, alpha-mangostin was the superior medicament in terms of viability reduction; it reduced the total CFE by 99.9% (Figure 4.9A). Moreover, the number of live cells was significantly reduced (p<0.0001): only 21.1% (1.03x10^5 CFE/mL) of bacterial and fungal cells remained after alpha-mangostin exposure compared to 9.75x10^7 CFE/mL in the saline control. HICA reduced the CFE by 99.8% (3.97x10^5 CFE/mL), with only 33.9% (3.41x10^5 CFE/mL) live microbial cells detected in biofilms after exposure (Figure 4.9A). The reduction in CFE and live cells after exposure to HICA and alpha-mangostin was significantly superior to both Calcicur® and Odontopaste® (p<0.05).

In deeper dentine samples, HICA and alpha-mangostin reduced CFE effectively by 99.9%, which was superior to Calcicur® and Odontopaste® (p<0.0001). However, the reduction of live cells in deeper dentine after exposure to HICA (71.1 % reduction) was significantly more than alpha-mangostin (p<0.0001) and the commercial medicaments (Figure 4.9B).

In the residual roots, the effectiveness of HICA and alpha-mangostin were in line with the other dentine depths. They reduced CFE effectively by up to 99.9%, which were superior to Calcicur® and Odontopaste® (p<0.0001) (Figure 4.9C). HICA also significantly reduced live cells compared to the others (p<0.0001), whereas Odontopaste® was the least effective medicament in terms of viability reduction at all dentine depths (Figure 4.9C).
4.4.5 Compositional analysis of a multi-species root canal biofilm by PMA-qPCR

All species-specific data showed the effectiveness of each medicament on in the biofilm composition (Figure 4.9A-C). In all dentine and residual root samples, S. gordonii comprised the major surviving species in biofilms, (10.2-40.5%), compared to the other species (Figure 4.9A). Even so, the proportion of S. gordonii in the biofilms of inner dentine samples was reduced from 40.2% to 23.0%, 13.3%, and 31.1% when exposed to HICA, alpha-mangostin, and Calcicur®, respectively (p<0.05). However, this proportion was increased to 69.8% when when biofilms were exposed to Odontopaste® (Figure 4.9A). Notably, the proportion of L. rhamnosus in the biofilm was reduced after exposure to HICA, alpha-mangostin, and Odontopaste®, but it was increased after exposure to with Calcicur®. The proportion of E. faecalis in the biofilms from inner dentine samples was similar following
exposure to all medicaments (2.0-2.9% viable *E. faecalis* were detected compared to 2.2% in saline control). In contrast to bacteria, the proportion of *C. albicans* detected in inner dentine samples was increased following exposure to all medications except HICA (Figure 4.9A).

In deeper dentine samples, the proportion of *S. gordonii* in the biofilm was significantly reduced after exposure to HICA (from 40.5% to 10.2%) (p<0.05) (Figure5.2B). In contrast, this proportion increased when these biofilms were exposed to Odontopaste® (from 40.5% to 55.1%) (p<0.05). The proportion of *C. albicans*, *E. faecalis*, and *L. rhamnosus* was reduced following exposure to any medication (Figure 4.9B).

In residual root samples, the proportion of *S. gordonii* in biofilms was significantly reduced (from 40.5% in controls to 11.2-33.9%) (p<0.05) when exposed to HICA, alpha-mangostin, and Calcicur®, but not with Odontopaste® (Figure 4.9C). The proportion of *C. albicans*, *E. faecalis*, and *L. rhamnosus* in biofilms was significantly reduced following exposure to all medicaments (Figure 4.9C).

4.5 Discussion

The development of a multi-species biofilm root canal model together with use of quantitative PMA-qPCR techniques provides a platform for understanding the interactions of individual species within biofilm. Moreover, the evaluation of novel root canal medicaments was accomplished *ex vivo* to mimic the clinical situation. Both culture and molecular methods showed that HICA and alpha-mangostin were superior to conventional calcium hydroxide or antibiotic-steroid medicaments in that they reduced the viability of candidal and bacterial within biofilms. Although there were still residual organisms following use of any medicament, only minimal quantities (0.1% of CFE/mL) were detected after use of HICA or alpha-mangostin compared to the current medicaments of choice. This initial study shows that these agents have the potential to be used as inter-appointment intra-canal medicaments in endodontic treatment.

This study investigated the efficacy of new reagents as potential medicaments in a root canal system mimicking the clinical scenario of bacterial and candidal biofilm infections during endodontic treatment. The major consideration was
microbial invasion into dentinal tubules; many factors including the nutritional environment, structure of dentine and cell adhesion play an important role (Brändle et al., 2008; Lang et al., 2010). Therefore, the inoculation period was set to 21 days to provide sufficient time for bacterial/fungal penetration and biofilm maturation in dentine tubules (Basrani et al., 2003; George et al., 2005; Zapata et al., 2008; Du et al., 2015; Sakko et al., 2017). In this study, the dentine excavated in the drilled samples showed that bacteria and Candida were able to migrate into the dentinal tubules. Moreover, HICA and alpha-mangostin were able to penetrate and effectively treat these infections compared to commercial medicaments.

In this study, we showed that saline was a suitable negative control because both bacteria and Candida grew after 7 days incubation. Calcium hydroxide (Calcicur®), the gold standard of intra-canal medicament, was used as a comparator in this study. The results showed the inferior antimicrobial activity of Calcicur® against multi-species biofilms compared to HICA and alpha-mangostin. This may be explained by the presence of two species in the biofilms which are resistant to calcium hydroxide: E. faecalis (Evans et al., 2002; Byström et al., 1985; Warren, 2013; Safavi et al., 1990; Ørstavik and Haapasalo, 1990; Sato et al., 1993; Weiger et al., 1995; Siqueira and de Uzeda, 1996; Tanriverdi et al., 1997) and C. albicans (Vaghela et al., 2011; Waltimo et al., 1999).

The antimicrobial activity of intra-canal medicaments may be naturally lower in the deeper dentinal tubules because of the decreasing in concentration of antimicrobials. Ex vivo studies have demonstrated that dentine can inactivate the antibacterial activity of Ca(OH)₂ (Haapasalo et al., 2000; Portenier et al., 2001). However, in this study, the inhibition of cell growth by Calcicur® was better in the deeper dentine depths. These findings may be explained by a water-based consistency of Calcicur® which can penetrate into the deeper dentine better than the other commercial zinc oxide-based Ca(OH)₂ paste. HICA and alpha-mangostin were still better than Calcicur® and perhaps this is so because they are also water-soluble.

The other comparator used in this study was Odontopaste®. This root canal medicament contains an antibiotic (clindamycin hydrochloride) and a steroid (triamcinolone acetonide). The antimicrobial activity of Odontopaste® against multi-species biofilms was inferior to HICA and alpha-mangostin. This may be because steroids can promote the growth of C. albicans (Kinsman et al., 1988; Guijar et al., 1997; Jainkittivong et al., 2007). Therefore, in this ex vivo model this medicament
was not suitable to inhibit *Candida* infections and may not be suited for persistent endodontic infections.

The molecular methods used in this study enabled rapid evaluation of the viability within the multi-species biofilms and their composition following various antimicrobial medicaments, including two novel candidates. The sensitive molecular approach by PCR methods revealed that residual viable organisms remained in the root canal system following all medicaments, which has significant clinical implications, although vastly fewer organisms remained following exposure to HICA and alpha-mangostin. PCR is a high sensitivity and specificity molecular method routinely used for identifying and quantifying oral microorganisms (Suzuki et al., 2004b; Park et al., 2011; Millhouse et al., 2014). However, using this technique alone does not distinguish between viable and dead cells because DNA can persist for an extended period of time following cell death (Young et al., 2007). The PMA-qPCR technique can be used to distinguish between viable from dead cells (Nocker and Camper, 2009; Loozen et al., 2011; Sanchez et al., 2013; Sanchez et al., 2014). In this study, the killing activity of root canal medicaments would have been overestimated if based on culture methodologies alone.

The composition analysis of the biofilms after exposure to root canal medicaments revealed that some organisms were more susceptible than others. *S. gordonii* was the most abundant organism remaining in all dentine depths following root canal medicaments. The dominance of facultative Gram-positive bacteria may be due to the harsh ecological conditions in the root canal after medication (Waltimo et al., 1997). The ability of *S. gordonii* to interact with other organisms in biofilms may confer this species an ecological advantage. The *Streptococcus* species are early colonisers of the oral cavity (Kolenbrander, 2011). *S. gordonii* can bind to *C. albicans* through streptococcal cell wall polysaccharide receptors (Holmes et al., 1995). However, further studies of the roles of *S. gordonii* in the root canal biofilms are required.

In addition, the compositional analysis showed that *C. albicans* accounted for only a small proportion of the root canal biofilm in comparison to the bacterial species. Previous studies have shown that candidal adhesion and hyphal formation were reduced when specific oral bacteria, including *P. gingivalis, Actinomyces* and
Streptococcus species were presented (Nair and Samaranayake, 1996; Vilchez et al., 2010; Guo et al., 2015). From this study, viable *E. faecalis* and *C. albicans* were detected at all dentine depths after medication with Calcicur®. The proportion of these species in roots was higher than when HICA or alpha-mangostin medicaments were used. This finding supported the results that both *E. faecalis* and *C. albicans* were resistant to calcium hydroxide (Evans et al., 2002; Waltimo et al., 1999).

### 4.6 Conclusion

The quantification and compositional analysis of multi-species root canal biofilms model provided information about the nature and quantity of each species within the biofilms. The effectiveness of various medicaments against root canal polymicrobial biofilms were demonstrated in terms of viable cell reduction and changes in population dynamics. The results of this study showed that both HICA and alpha-mangostin exerted superior activity in reducing biofilm viability and they have the potential to be used as root canal medicaments, especially for persistent infections or retreatment cases.
4.7 References


Chapter 5

General Discussion
5.1 Study Overview

The use of antimicrobial agents and antiseptics is a part of endodontic treatment. These agents must be effective and biocompatible. This series of studies was performed to gain insight into the antimicrobial spectrum of HICA and alpha-mangostin as well as their potential for root canal medication. Many *in vitro* studies have demonstrated the susceptibility of these agents against a wide range of planktonic microorganisms including bacteria and fungi. However, no studies have been carried out on multi-species biofilms. The overall aim of this thesis was accomplished by investigating the antimicrobial properties of novel and alternative root canal medicaments on bacterial, fungal and multi-species biofilms in a model that mimics a clinical setting.

This thesis has three distinct concerns: firstly, the susceptibility of both planktonic bacteria and yeast to HICA and alpha-mangostin was confirmed and served the purpose of determining concentrations to be used in further studies. XTT assays showed that HICA and alpha-mangostin suppressed metabolism of bacterial, fungal and multi-species biofilms. In addition, bright field and Baclight™ LIVE/DEAD staining showed that these agents altered the biofilm structure. Culture and staining techniques quantified biofilm viability and offered an insight into some of the difficulties of using these techniques (Chapter 2,3). Secondly, time-kill assays determined that the antimicrobial activity of HICA and alpha-mangostin against planktonic root canal microbes was more effective compared to commercial root canal medicaments (Chapter 4). Moreover, the inhibitory capability of HICA and alpha-mangostin was shown in an *ex vivo* root canal multi-species biofilms model (Chapter 4). Finally, sensitive molecular methods measured survival and composition within the root canal biofilms (Chapter 4). The infection dynamics revealed that in this setting, *S. gordonii* was the main survivor in root canal biofilms and HICA was best at eradicating growth.

The hypothesis that HICA and alpha-mangostin are potential antimicrobial agents for the treatment of root canal infections was confirmed in this thesis. The results indicate that both agents have antimicrobial activity against several species of oral bacteria species and *Candida*, separately in planktonic and biofilm modes of growth and against combined multi-species biofilms. Both HICA and alpha-
mangostin showed superior inhibitory activity against established biofilms in infected human dental root canals compared to conventional root canal medicaments.

5.2 Methodological considerations

Multi-species fungal-bacterial biofilm models are increasingly valuable investigative tools. Growing multi-species biofilm \textit{in vitro} is challenging, reproducibility is crucial to compare different growth conditions or inhibitory substances at different concentrations (Brändle et al., 2008; Yamazaki et al., 2011; Ramalingam et al., 2012; Muhammad et al., 2014; Salli and Ouwehand, 2015; Rahmani-Badi et al., 2015; Soares et al., 2015). A number of methods been developed for testing efficacy of various antimicrobial agents on biofilm. These are based on growing microorganisms on tubing, plastic pins, glass rods, or in wells using a constant flow of media, all providing surfaces for biofilm growth (Vickery et. al., 2004; Loukili et. al., 2004; Parker et. al., 2014). Biofilm detection methods range from counting colony forming units to microscopy and the use of crystal violet (Xu et. al., 2016). Biofilm viability can be measured by fluorescent staining techniques or by culture. The metabolic activity is commonly measured by XTT. Alternatively, a CDC reactor can be used to grow biofilms on silicone, and the biofilm biomass can be further quantified (Honraet et al., 2005). Moreover, the MBEC system is the other method to grow biofilms on the plastic surface peg instead of a flat cover slip (Parker et. al., 2014). Considering the MBEC system in particular, the growth of the biofilm is much more difficult to visualise on a small cylinder shaped (rolling) peg and the surface area of a small peg would not provide enough biomass for multiple tests to be performed, so the MBEC may not be ideal for this model. The benefit of the MBEC system is that it allows high throughput testing of hundreds isolates as required in product development (Ceri et. al., 2001), but with the coverslips, imaging is easier and biofilm biomass is bigger and it is more suitable for scientific experiments.

This study is the largest study of the antimicrobial spectrum of HICA and alpha-mangostin in a multi-species biofilms model. It is the first study to perform compositional analysis of root canal biofilms \textit{ex vivo}. The experiments were performed using biofilms which were grown on coverslip or well-plates \textit{in vitro}.
(Chapter 2, 3) and in human root canals ex vivo (Chapter 4). In addition to culture methods, a sensitive molecular technique was used to evaluate quantity and composition within biofilms (Chapter 4) (Sherry et al., 2016; Sánchez et al., 2014). The selected methods used in this proof of concept study were suitable to assess the use of new antiseptics in the root canal treatment. The ex vivo biofilm model with the molecular technique will provide useful data for the clinical trials.

5.2.1 Culture media

A culture medium is an essential prerequisite for microbial growth. Different media suitable for methodology were tested in this study. For the susceptibility testing of aerobic and facultative bacteria, Mueller-Hinton broth was used (Wikler, 2009), while RPMI-1640 was used for Candida species (CLSI, 2008). The results of the present studies are comparable to other similar microbiological studies in the development of new antimicrobial agents (Sakko et al., 2012; Sakko et al., 2014). Brain heart infusion broth was used to incubate microbes into root canals. The use of this media was adapted from a similar root canal infection model in which microbes were incubated for three weeks (Basrani et al., 2002; Basrani et al., 2003; Sakko et al., 2017). There is evidence to show bacterial penetration into dentine tubules using this media (Basrani et al., 2003; Zapata et al., 2008; Du et al., 2015; Sakko et al., 2017).

5.2.2 Negative controls and positive comparators

Negative controls were used for control of microbial growth in media, they allowed microbes to grow freely in the nutrient-rich conditions. The negative control allowed passive reduction in microbial viability in nutrient-deficient conditions including infected root canal. The nutrient rich media are required in planktonic settings to conform to the standards required for susceptibility testing. Therefore, Mueller-Hinton broth and RPMI-1640 media were used in the susceptibility experiments, and RPMI-1640 media was used in the XTT assays. However, the sterile saline was used as a negative control in the ex vivo tooth model to mimic the nutrient-deficient environment in root canals during the endodontic treatment (Sundqvist and Figdor, 2003).
The comparators were used for microbiocidal control. NaOCl and CHX which are the standard endodontic irrigants, were used in the XTT experiments. In addition, the commercial root canal medicaments (Calcicur® and Odontopaste®) were used in the ex vivo tooth model. These two materials were paste-like in consistency; they can be retained in the root canals over 7-days of medication.

### 5.2.3 Test organisms and microbial cultures

For evaluating the microbial spectrum of HICA and alpha-mangostin, pathogenic organisms were chosen as representatives of microbial species commonly found in the persistent root canal infection, and that both clinical and reference isolates were used in separate and in multi-species combinations. The antimicrobial activity of these agents on selected isolates can be implied clinically to persistent endodontic infections.

Planktonic organisms were exposed to antimicrobials in the susceptibility tests and time-kill assays. The incubation time was 48 hours to allow sufficient growth of organisms. ICs, MBCs, and MFCs were detected following the breakpoints of standard methods. In the ex vivo tooth model, three weeks of incubation was suitable to allow the biofilm maturation in root canal dentine as shown in the results of control biofilms, this was in line with previous studies (Zapata et al., 2008; Du et al., 2015; Sakko et al., 2017).

### 5.2.4 Group size and statistical analysis

The number of replicates and repeats in the experiments should have been larger to substantiate statistical analyses. For the XTT assays, the experiments were carried out twice in triplicate. Therefore, the data were sufficient for statistical analysis. In the ex vivo tooth model experiment, the size of the test and control groups were larger with the number of replicates per group being 20 and one repeat could be performed. Three isolates of bacteria and one of candida isolate were used as a technical replicate in all incubations. Different human teeth represented true biological replicates in each incubation. Statistical analyses were performed appropriately in the present study.
5.3 Antimicrobial activity and suitability for root canal medication

5.3.1 HICA

The present study was the first study of the impact HICA on bacterial, fungal, and multi-species biofilms. The metabolic activity and the viable cells within biofilms were significantly reduced after exposure to HICA for 24 hours. The activity of HICA against various root canal microbes is favourable for endodontic use, HICA killed the planktonic organisms within 24 hours in the time-kill assays. Previous studies on the impact of biological factors such as dentine, hydroxyapatite, and serum albumin on the antibacterial activity of HICA showed that increasing the concentration of HICA provided greater tolerance to interactions with these factors (Sakko et al., 2016). Therefore, higher concentration of HICA (400,000 mg/L) which is 10-fold the MBC against E. faecalis were chosen for the observations in the experimental infection root canals study (Sakko et al., 2017). Moreover, the 400,000 mg/L of HICA is a paste-like consistency which is suitable for the delivery and long retention in root canals.

In the *ex vivo* multi-species biofilms experiment, the antimicrobial activity of HICA was higher than the commercial root canal medicaments at all dentine depths. For the quantitative comparisons, the culture technique was in line with qPCR. Significant reduction of viable bacterial and candidal cells was achieved with HICA treatment in comparison with calcium hydroxide paste and antibiotic-steroid containing root canal medicaments. The compositional analysis demonstrated the reduction in the proportion of all microbes within biofilms after the treatment. The activities of HICA to reduce biofilm biomass and viable cells were comparable or better than currently-used inter-appointment medicaments in endodontology, it could be a promising agent for root canal medication.

5.3.2 Alpha-mangostin

The present study was the first to assess the anti-biofilm activity of alpha-mangostin. The metabolic activity and viable cells within biofilms was significantly reduced after exposure to alpha-mangostin for 24 hours. The anti-biofilm activity of alpha-mangostin was comparable to 20,000 mg/L CHX. The activity of alpha-mangostin against various root canal isolates is favourable for endodontic use alpha-mangostin killed all tested planktonic species within 72 hours.
In the *ex vivo* multi-species biofilms experiment, the antimicrobial activity of alpha-mangostin was higher than the commercial root canal medicaments at all dentine depths. The compositional analysis also demonstrated the reduction in the proportion of all microbes within biofilms. Alpha-mangostin is a small compound with 410.466 g/mol of molecular weight (PubChem CID: 5281650), it could easily penetrate into dentinal tubules.

5.3.3 Calcium hydroxide

According to quantitative analyses in this study, Ca(OH)$_2$ was able to inhibit cell growth within biofilms. However, both viable bacterial and candidal cells were detected following exposure to medicaments. This finding is in line with previous studies in which bacterial growth was detected in more than 90% of Ca(OH)$_2$- medicated root canals (Basrani et al., 2002; Basrani et al., 2003). The compositional analysis in this study demonstrated that the viable *C. albicans* and *E. faecalis* were remained in the roots with Ca(OH)$_2$ medicament. This confirms the previous finding that both *C. albicans* and *E. faecalis* were resist to Ca(OH)$_2$ (Ercan et al., 2006).

5.3.4 Antibiotic-steroid containing root canal medicament

The results from this study showed that Odontopaste$^\text{®}$ inhibited cell growth within biofilms better Ca(OH)$_2$ paste, but the activity was inferior to HICA and alpha-mangostin. The bactericidal activity of this medicament is due to the antibiotic component. Clindamycin is a broad-spectrum antibiotic that is effective against a wide range of microorganisms including aerobic, anaerobic, and $\beta$-lactamase-producing pathogens (Brook et al., 2005). However, resistance of *E. faecalis* to clindamycin has been reported (Singh and Murray, 2005; Huycke et al., 1998).

The quantitative analyses in this study showed that the ability of Odontopaste$^\text{®}$ to reduced biofilm biomass was better than Ca(OH)$_2$ paste. However, the proportion of *C. albicans* was significantly increased after medication with Odontopaste$^\text{®}$. This supported the findings that the corticosteroid component in Odontopaste$^\text{®}$ can promote the growth of *C. albicans* (Kinsman et al., 1988; Gujjar et al., 1997; Jainkittivong et al., 2007).
5.4 Future work

The current study has enabled a better understanding of the growth and dynamics of multi-species bacterial-fungal biofilms in the root canal environment. Further analysis of the impact of both HICA and alpha-mangostin on the biofilm structure would allow a more detailed understanding of the potential effect of medicaments on each organism within the biofilm at various dentine depths. The penetration of each species into the dentinal tubules and dentine disinfection analysis could be performed by using confocal laser scanning microscopy (Ma et al., 2011; Parmar et al., 2011; Wood et al., 2000; Wang et al., 2012).

The safety profile of root canal medicaments is one of the major considerations because of the potential for contact with periodontal cells (Ruparel et al., 2012). The safety of HICA and alpha-mangostin has been extensively studied, and HICA has been used systemically (Ojala et al., 2014; Bagchi et al., 2013). Moreover, the potential interactions between HICA and alpha-mangostin with other agents and chemicals used in endodontics and dentistry should be assessed.

Further screening of a larger number of isolates following the relevant regulatory standards is needed before it is possible to move on to clinical trials. For the relevant legislation, standards and guidance linked to provide supporting evidence for new antimicrobial/disinfectant medicaments is required. The EMA is one of the organisations which has set standards for the quality, safety and efficacy of authorised medicines, primarily laid down in Directive 2001/83/EC and in Regulation (EC) No 726/2004. An alternative approach could have been via the US FDA technical monographs. In terms of demonstrating efficacy evidence for new antimicrobial medicaments, EN 14885: Chemical disinfectants and antiseptics - Application of European Standards for chemical disinfectants and antiseptics, BS EN 1040 Basic bactericidal activity and BS EN 1276 Quantitative bacterial suspension test is recommended. To put a medical device or antiseptics on the European market, the intended purpose of the product must be defined by the manufacturer to be used for human beings for the purpose of diagnosis, prevention, monitoring, treatment or alleviation of disease. The clinical efficacy and any side effects, if applicable by means of a pre-clinical and clinical evaluation must be evaluated (French-Mowat & Burnett, 2012). A conformity assessment procedure demonstrates that the medical device complies with the requirements of Directive 93/42/EEC.
HICA and alpha-mangostin are classified as class III and therefore pre-market approval is required to ensure the safety and effectiveness of these agents.

The antimicrobial activity and efficacy of HICA and alpha-mangostin as root canal medicaments should be assessed next in a clinical setting in a randomised controlled clinical trial (Molander et al., 2007; Penesis et al., 2008; Paredes-Vieyra and Enríquez, 2012). The activity of HICA and alpha-mangostin should be compared with the standard intra-canal medicament. Based on the ex vivo experiments, concentrations ten-fold or higher than planktonic MICs should be used. For infections caused by a mixture of microbes such as used in this study, 400,000 mg/L HICA and 10,000 mg/L alpha-mangostin should be effective. In clinical studies, seven days is the most widely used and accepted time between appointments for the root canal medication (Kim and Kim, 2015; Sathorn et al., 2007). To evaluate the microbial activity of the medicaments, the microbiological samples from root canals should be collected after inter-appointment medication. The most commonly used sampling method is sterile paper point placed into the canal for one minute after gently filing root canal walls (Vianna et al., 2007; Rôças and Siqueira, 2011; Paiva et al., 2013; Sinha et al., 2013). Burs such as those used in the present ex vivo study cannot be used in clinical setting but enlarging the canal with the next size rotary file would be possible and is in fact common practice as mechanical preparation is essential for the elimination of microbes as well as preparing the root canal space for further obturation (Stewart, 1955). It would be important to use the same method of instrumentation and sampling throughout the study and for all patients. Also, to avoid bias only teeth with single root and single root canal should be included and teeth with previous endodontic treatment should be excluded.

In clinical practice, the quantification of root canal microbes relies on culture-based techniques. However, the majority of microorganisms cannot be cultured with standard laboratory techniques (Amann et al., 1995; Pace, 1997). Only an approximate quantification can be evaluated by counting colony forming units (CFU). To provide a more accurate determination of the total microbial content in the root canal systems, molecular methods for the identification and characterisation of microbial communities are warranted (Ginzinger, 2002). The real-time quantitative polymerase chain reaction (RT-qPCR) with primers directed against the bacterial 16S ribosomal RNA (rRNA) or fungal 18S rRNA genes is highly sensitive and
precise. This study showed it was effective to quantify root canal microbes and could be the method used in the clinical trial.

Apart from microbial reduction after medication, the healing of apical periodontitis can be used as a parameter of achievement of root canal treatment (Molander et al., 2007). Clinical signs and symptoms should also be followed during and after the treatment. Moreover, the periapical index (PAI) scoring method could be used to evaluate radiological changes (Ørstavik et al., 1986). According to guidelines for endodontic treatment from European Society of Endodontontology, all patients should be followed-up for four years to observe the healing of endodontic diseases (Loest, 2006). It is important to define clearly the criteria for treatment success for the clinical trial. In 4% of root canal treatments done using best clinical practice, radiological improvement cannot be seen (Ng et al., 2007).

The determination of the optimal sample size for a clinical research study requires an adequate power to detect statistical significance. High values of power (at least 80%) are desirable to give the available resources and ethical considerations (Suresh and Chandrashekara, 2012). Assuming that the efficacy of HICA and alpha-mangostin will be similar to that seen in the ex vivo study and that the clinical and radiological responses correlate with microbiological response, a sample size of 18 per group should be powered to provide evidence of clinical efficacy. Taking into consideration the risk for patients dropping out of the trial or teeth being lost due to fractures or other incidents, it is likely that 20-25 patients will need to be recruited.

5.5 Conclusion

As discussed through this thesis, the antimicrobial spectrum of HICA and alpha-mangostin has been established in single and multi-species biofilms in experimentally-infected dental root canals. The hypotheses that HICA and alpha-mangostin could be a useful antimicrobial agent for the treatment topical infections including root canal infections were accepted. The results of the present investigation indicate that HICA and alpha-mangostin were clearly microbiocidal for all organisms tested, they also have anti-biofilm activity against bacterial, fungal, and multi-species biofilms. Both agents have antimicrobial activity against multi-species biofilms in human dental root canals, they were more active than Ca(OH)$_2$
medicament in terms of biofilm biomass and viability reduction in various dentine depths.

The major factors associated with endodontic failure are the persistence of microbial infection in the root canal systems as microbes tolerate to antimicrobial agents (Siqueira, 2001; Nair et al., 1990; Lin et al., 1992). The results of the present study indicate that HICA and alpha-mangostin were active against the causative organisms of persistent root canal infections in an ex vivo model mimicking these infections. The biocompatibility of HICA and alpha-mangostin should be tested before clinical studies, because it is possible for the medicaments to extrude or leak from the root canal system into periapical and periodontal tissue tissues. Further in vivo studies are necessary to evaluate the effect of HICA and alpha-mangostin as a root canal medicament.

The present investigation in human dental root canals provides basic evidence of antimicrobial potential of HICA and alpha-mangostin for further investigations in their use in endodontiology and the management of other topical infections.
5.6 References


