Marjut Sakko

ANTIMICROBIAL ACTIVITY AND SUITABILITY OF 2-HYDROXYISOCAPROIC ACID FOR THE TREATMENT OF ROOT CANAL INFECTIONS
ACTA UNIVERSITATIS OULUENSIS
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MARJUT SAKKO

ANTIMICROBIAL ACTIVITY AND SUITABILITY OF 2-HYDROXYISOCAPROIC ACID FOR THE TREATMENT OF ROOT CANAL INFECTIONS

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Lecture Hall 3, Biomedical Helsinki 1 (Haartmaninkatu 8, Helsinki), on 26 February 2016, at 12 noon

UNIVERSITY OF OULU, OULU 2016
Abstract

Microbial infection in dental root canal induces an inflammatory reaction called apical periodontitis. In post-treatment disease, *Enterococcus faecalis* is the most commonly found organism, which may survive well in root canal despite calcium hydroxide paste medication. In these cases, effective irrigation or repeated chlorhexidine medication are recommended. New medications with long-lasting antimicrobial activity are needed for the treatment of persistent root canal infections. 2-hydroxyisocaproic acid (HICA) is a protein fermentation product of lactobacilli and few other bacterial or fungal species express enzymes required for its metabolism. However, mammalian cells can metabolise it and use it for protein production. It is known to be well-tolerated by humans and have anti-inflammatory properties as well. Therefore, the hypothesis was that it affects microbe-specific metabolic pathways and have potential as a novel antimicrobial agent.

The aim of this study was to evaluate the antibacterial and antifungal spectrum of HICA using *in vitro* microdilution methods for susceptibility testing and an *ex vivo* extracted tooth root infection-model. The impact of dentine on the antimicrobial activity of HICA was also evaluated. The results showed that HICA has broad-spectrum bactericidal activity for gram-positive and gram-negative organisms. It is also fungicidal for several fungal species and it is only marginally inactivated by clinically-relevant concentrations of dentine. It is at least as active against *E. faecalis* as presently-used interappointment medications in infected root canals after a seven-day incubation *ex vivo*. Since HICA has a broad-spectrum and long-term antimicrobial activity as well as an anti-inflammatory effect, it may be a useful new agent for clinical endodontology. However, controlled clinical studies are needed for evaluation of the efficacy of HICA in clinical conditions.

Keywords: 2-hydroxyisocaproic acid, antimicrobial, apical periodontitis, antiseptic agent, calcium hydroxide, chlorhexidine, disinfectant, root canal medication
Sakko, Marjut, 2-hydroksi-isokapronihappo antimikrobiteho ja soveltuvuus juurikanavainfektioihin.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Finnish Doctoral Program in Oral Sciences

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


*Lactobacillus*-bakteerit tuottavat proteiiniainenvaihdunnassa 2-hydroksi-isokapronihappoa eli HICAa, jonka metaboloimiseen tarvittavia entsyymejä tunnetaan vain muutamilla muilla bakteereilla. Ihmisillä metaboloivat ja käyttävät sitä proteiinituotannoon. Se on hyvin siedetty aine ja se lieventää tulehdusreaktiota. Tutkimuksen hypoteesina oli, että HICA vaikuttaa mikrobiin annekseen ja se voisi olla mahdollinen uusi antimikrobiaine.


Asiasanat: 2-hydroksi-isokapronihappo, antimikrobiaine, apikaaliparodontiitti, antiseptinen aine, desinfiointiaine, juurikanavalääke, kalsiumhydroksidi, klooriheksidiini
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Tuusula, December 2015

Marjut Sakko
### Abbreviations

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<tr>
<td>AP</td>
<td>apical periodontitis</td>
</tr>
<tr>
<td>ASM</td>
<td>American Society of Microbiology</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BHI</td>
<td>brain heart infusion</td>
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<td>BRU</td>
<td>brucella agar</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca(OH)$_2$</td>
<td>calcium hydroxide</td>
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<tr>
<td>CCUG</td>
<td>Culture Collection of the University of Gothenburg</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CHA</td>
<td>chlorhexidine acetate</td>
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<tr>
<td>CHG</td>
<td>chlorhexidine digluconate</td>
</tr>
<tr>
<td>CHX</td>
<td>chlorhexidine</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>CMCP</td>
<td>camphorated paramonochlorophenol</td>
</tr>
<tr>
<td>CP</td>
<td>camphorated phenol</td>
</tr>
<tr>
<td>DL</td>
<td>dextro and levo-enantiomers</td>
</tr>
<tr>
<td>DP</td>
<td>dentine powder</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission of International Union of Biochemistry and Molecular Biology</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>ESBL</td>
<td>extended spectrum of beta-lactamase</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<tr>
<td>FAA</td>
<td>fastidious anaerobe agar</td>
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<tr>
<td>FLU</td>
<td>fluconazole</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
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<td>HICA</td>
<td>2-hydroxyisocaproic acid</td>
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<tr>
<td>HicDH</td>
<td>hydroxyisocaproic acid dehydrogenase</td>
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<tr>
<td>IC</td>
<td>inhibitory concentration</td>
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<tr>
<td>IPI</td>
<td>iodine potassium iodine</td>
</tr>
<tr>
<td>ITR</td>
<td>itraconazole</td>
</tr>
<tr>
<td>KICA</td>
<td>2-ketoisocaproic acid</td>
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<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MBC</td>
<td>minimum bactericidal concentration</td>
</tr>
<tr>
<td>MRCM</td>
<td>Mycology Reference Centre Manchester</td>
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<tr>
<td>MRSA</td>
<td>methicillin-resistant \textit{Staphylococcus aureus}</td>
</tr>
<tr>
<td>MSUD</td>
<td>maple syrup urine disease</td>
</tr>
<tr>
<td>NaOCl</td>
<td>sodium hypochlorite</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NGS</td>
<td>next-generation DNA sequencing</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leucocytes</td>
</tr>
<tr>
<td>PW</td>
<td>peptone water</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SAB</td>
<td>Sabouraud agar</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptic soya agar</td>
</tr>
<tr>
<td>UK NEQAS</td>
<td>United Kingdom National External Quality Assessment Service</td>
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List of original publications

This thesis is based on the following publications, which are referred throughout the text by Roman numerals:


The original publications have been reprinted with permission of copyright holders. In addition, this thesis contains unpublished data.
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1 Introduction

In general, microbial resistance to antimicrobials cause severe problems in the treatment of infections. The excessive use of systemic antibiotics further increase the emerging problem (World Health Organization 2014). Microbial colonisation is generally seen as a prerequisite for a topical or systemic infection. Prevention of colonisation in order to prevent infection process would be a useful target for managing infection.

In the management of superficial infections, topical delivery of antimicrobial agents may have several advantages in comparison to systemic use. At a site of infection, high topical concentrations are easily achieved and the risk of systemic toxicity is reduced. In addition, selection of species resistant to systemic antimicrobials would decrease with the use of novel agents with different antimicrobial mechanisms from the mode of action of current antimicrobials in use and the use of antibiotics could be limited for management of severe deep infections. In the development of new antimicrobials those agents unsuitable for systemic treatments could be considered for topical use (McHugh et al. 2011).

Topical antimicrobials are the first line medications in the treatment of dental root canal infections. Persisting root canal infection is the main reason for apical periodontitis (AP) in post-treatment infections (Peciuliene et al. 2008, Siqueira & Rôças 2014). Microbial biofilms, which are associated with AP, have an increased ability to resist the effect of antimicrobials in comparison to free-floating cells (Ceri et al. 1999, Lang et al. 2012).

The strategy of root canal treatment is based on microbial eradication from root canals by both mechanical and chemical means, while interappointment medication does not necessarily lead to significantly better treatment outcomes than single-visit treatment (Weiger et al. 2000, Shuping et al. 2000, Kvist et al. 2004, Vivacqua-Gomes et al. 2005, Paredes-Vieyra & Enriquez 2012, Vera et al. 2012). Unreachable sites of complex root canal anatomy by mechanical instrumentation or disinfection are potential niches for microbial persistence.

Highly alkaline calcium hydroxide (Ca(OH)\textsubscript{2}) is the most generally-used agent in interappointment medication, but there are conflicting results regarding its antimicrobial activity (Ørstavik et al. 1991, Reit et al. 1999, Peters et al. 2002, Vera et al. 2012, Kim & Kim 2014, 2015). Ca(OH)\textsubscript{2}-resistant strains may cause difficult-to-treat root canal infections. Chlorhexidine, sodium hypochlorite, and iodine potassium iodine all have a different spectrum of activity in comparison to Ca(OH)\textsubscript{2}. Despite their many positive properties in endodontic treatment, short-term activity,
potential of tissue irritation, allergic and toxic reaction caused by these agents restrict their wider use as intracanal interappointment medication (Fedorowitz et al. 2012, Guleri et al. 2012, Pasternak & Williamson 2012).

Inorganic and organic content in root canals is reported to reduce antimicrobial activity of disinfectants (Haapasalo et al. 2000, Portenier et al. 2001, 2002, 2006, Oliveira de et al. 2010). In contrast, dentine extracellular matrix is shown to have antimicrobial activity (Smith et al. 2012). New safer and more active antimicrobials in comparison to presently-used agents are needed for achieving better treatment outcomes in endodontology.

2-hydroxyisocaproic acid (HICA) could be a new and safe topical antimicrobial agent, since it has antimicrobial properties (Hietala et al. 1979), it is naturally found in nutrition (European Food Safety Authority 2012), and it is metabolised by human cells (Hoffer et al. 1993, Dallmann et al. 2012). HICA is a bacterial protein fermentation product, which is a by-product of the leucine-acetyl-CoA pathway in the leucine degradation pathway. For HICA elimination hydroxyisocaproic acid dehydrogenase (HicDH) enzyme is needed. Both are mainly produced by lactobacilli (Hummel et al. 1985, Yamakazi & Maeda 1986, Hummel & Kula 1989, Kallwass 1992, Bernard et al. 1994, Niefind et al. 1994, 1995, Dengler et al. 1997, Yvon & Rijnen 2001, Broadbent et al. 2004, Smit et al. 2004, 2005, Chambellon et al. 2009).

The present series of studies focuses on the antimicrobial spectrum of HICA. Antibacterial activity of HICA was observed for a spectrum of human pathogenic facultatively and obligately anaerobic gram-positive and gram-negative bacterial species. The antibiotic-resistant strains of methicillin-resistant Staphylococcus aureus (MRSA) and Escherichia coli with extended-spectrum beta-lactamase (ESBL) were included. The antifungal spectrum of Candida and Aspergillus species for HICA and azoles was tested. The impact of HICA on hyphal formation of Candida albicans and filamentation of Aspergillus fumigatus, which are associated with the pathogenesis and virulence, were evaluated.

A particular interest of this study was the potential of HICA for root canal medication. Enterococcus faecalis has been the most common finding in persisting root canal infections. The interactions between HICA and potential inhibitors such as dentine, hydroxyapatite, and serum albumin were examined in 48 h incubation. Longevity of antibacterial activity of HICA against E. faecalis 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.
The hypothesis of this study was that HICA is a potential antimicrobial agent for the treatment of topical infections. The purpose of this study was to evaluate the antimicrobial spectrum and the suitability of HICA for the management of topical infections in dental root canals.
2 Review of the literature

2.1 2-hydroxyisocaproic acid (HICA)

2.1.1 Distribution and biochemistry

HICA is also known as leucic acid, 2-hydroxy-4-methylvaleric acid, or 2-hydroxy-4-methylpentanoic acid, with a molecular weight of 132.16 g/mol (Takayama 1933). It is patented for antimicrobial and anti-inflammatory uses (Westermarck et al. 2008; EP 0871438 B1, “Use of alpha-hydroxy acids in the manufacture of a medicament for the treatment of inflammation”). HICA is a by-product of the leucine-acetyl-CoA pathway (Fig. 1). Together with isoleucine and valine, leucine is one of the three essential branched-chain amino acids (BCAA) in humans, which is primarily acquired from nutrition. HICA is produced in animal protein fermentation of *Lactobacillus plantarum* (Hietala et al. 1979). The other organisms, which are able to produce HICA are *Clostridium difficile* (Brooks et al. 1984), other lactobacilli (Hummel et al. 1985, Bernard et al. 1994, Yvon & Rijnen 2001, Chambellon et al. 2009), *Peptostreptococcus anaerobius* (Hamid et al. 1997), and *Weissenella paramesenteroides* (Ndagano et al. 2011). Some environmental bacteria of *Bacillus*, *Pseudomonas*, *Streptomyces* species, and the *Coryneform* group are also able to produce HICA by enantiospecific dehydrogenation of different racemic aliphatic 2-hydroxyacids (Sawada et al. 1983).

HICA is formed by transamination of leucine to 2-ketoisocaproic acid (KICA), which is followed by reduction of KICA to HICA (Fig. 1; Smit et al. 2004). Both breakdown products are incorporated into normal biochemical pathways to energy production in the citric acid cycle (Kanehisa et al. 2014). In yeast, these reactions of the leucine metabolic pathway seem to be cytocolic reactions (Kohlaw 2003). Aminotransferase enzymes (EC 2.6.1.42, EC 2.6.1.6) in conversion of leucine to KICA have been described in a number of micro-organisms (Kanehisa et al. 2014). HICA is thought to be the end product of KICA reduction (Hoffer et al. 1993, Yvon & Rijnen 2001, Chambellon et al. 2009). Therefore, conversion by oxidation reaction from HICA back to KICA is required for HICA elimination (Bossw & Wandrey 2006). The reduction reaction is catalyzed by hydroxyisocaproic acid dehydrogenase (Fig. 1; HicDH: EC 1.1.1.-), which is mainly detected in lactic acid bacteria (Hummel et al. 1985, Yamakazi & Maeda 1986, Hummel & Kula 1989, Kallwass 1992, Bernard et al. 1994, Niefind et al. 1994, Niefind et al. 1995,

Fig. 1. A schematic illustration of leucine degradation pathway.

**Hydroxyisocaproic acid dehydrogenase**

HicDH enzymes belong to a subgroup of lactate dehydrogenases (LDH) in the family of 2-hydroxycarboxylate dehydrogenases (Dengler et al. 1997). They catalyse NAD-dependent reversible stereospecific interconversion of D- or L-isomers between 2-ketocarboxylic and 2-hydroxycarboxylic acids (Schütte et al. 1984, Smit et al. 2005). D-HicDH and D-LDH have a similar protein structure, although their relationship is not clear fully (Yamakazi & Maeda 1986, Tamura et al. 2002). Conversion of LDH to HicDH is also shown in lactobacilli (Tokuda et al. 2003). Another enzyme from the LDH subgroup is D-mandelate dehydrogenase, which has been isolated from *E. faecalis* (Tamura et al. 2002). The enzymes in the LDH subgroup are named after their main substrates, but crossreactions between these enzymes and the substrates may occur (Yamakazi & Maeda 1986, Tamura et al. 2002). The activity of HicDH enzyme differs between bacterial species and isolates (Bachmann et al. 2009). The activity is regulated by *panE* gene. Inactivation of this gene leads to reduction of HICA production in *Lactococcus lactis* (Cadiñanos de et al. 2013). The D-HicDH coding gene is also detected in *E.*

**HICA in humans**

The reactions of BCAAs and their derivatives take place mainly in muscles, liver, brain, and kidneys (Hoffer et al. 1993, Suryawan et al. 1998, Holeczek 2002). The BCAA aminotransferases are both mitochondrial and cytosolic (Wu et al. 2004). HICA is metabolised in an analogous manner in the leucine degradation pathway in humans. It is isolated from human plasma (Dallmann et al. 2012), urine (Tanaka et al. 1980), and amniotic fluid (Jacobs et al. 1984). Plasma concentration of HICA (0.1–0.25 μmol/L) is normally 30–100 times lower than KICA, while it increases during prolonged fasting and after exercise due to use of proteins as an energy source. HicDH activity may also be bidirectional in humans. (Hoffer et al. 1993).

Defects in branched-chain keto acid dehydrogenase complex (EC 1.2.4.4) lead to accumulation of several BCAA metabolites including HICA and odor of urine. This is called maple syrup urine disease (MSUD). Unless diagnosed early in newborns, it may cause extensive encephalopathic symptoms mainly because of increased amounts of leucine and 2-keto acids. Oxidative stress is supposed to participate in the neuropathology. (Mamer & Reimer 1992, Fontella et al. 2002, Heldt et al. 2005, Frazier et al. 2014). Elevated HICA concentrations are also found in diabetic ketoacidosis (Lieblich & Först 1984), in dihydrolipoyl dehydrogenase deficiency (EC 1.8.1.4) (Haan et al. 1985), and in short bowel syndrome (Kuhara et al. 1983). Deficiency of dihydrolipoyl dehydrogenase leads to atypical MSUD and in short bowel syndrome an increased amount of lactobacilli in gut flora is also seen (Haan et al. 1985). It is suggested that the D-enantiomer of HICA from bacterial origin dominates in urine in short bowel syndrome and L-form from human origin in MSUD (Spaapen et al. 1987).

**2.1.2 Antimicrobial properties**

Secondary metabolites of many lactic acid bacteria possess broad-spectrum antimicrobial properties. Inter alia, Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus, Weissenella, and Enterococcus species belong to this group of bacteria, which produce lactic acid as a major end product of carbohydrate metabolism. Antimicrobial effects of microbial production of organic acids, hydrogen peroxide, and bacteriocins have been widely examined (Olasupo 1996,
Ricke 2003, Todorov 2009). Antimicrobial compounds of phenyllactic acid, cyclic dipeptides (Ström et al. 2002), peptides (Brogden et al. 2005, Hayes et al. 2006, Gupta & Srivastava 2014), and 3-hydroxy fatty acids (Broberg et al. 2007) have also been investigated. Hietala et al. (1979) also reported antimicrobial properties of lactic acid and 2-hydroxypropionic acid. Mandelic acid or 2-hydroxy-2-phenylacetic acid have been proposed for the treatment of urogenital infections (Putten van 1979, Zaneveld et al. 2002). Antimicrobial peptides have recently also been suggested for root canal medication (Lee et al. 2013, Freitas de Lima et al. 2015, Wang et al. 2015).

HICA has antibacterial activity against E. coli (Hietala et al. 1979), which is the most general aerobic bacteria of human intestinal microflora and causes mainly urogenital infections, enteric infections and enteritis. Antifungal activity of HICA has been shown against Trichophyton (Hietala et al. 1979), Aspergillus and Penicillium species (Broberg et al. 2007, Ndagano et al. 2011) and against C. albicans biofilms (Nieminan et al. 2014a). C. albicans is the most common fungus in human oral microflora (Ghanoum et al. 2010) and the most general opportunistic pathogen in superficial and deep candidosis (Jones et al. 2004, Arana et al. 2009). Aspergillus and Penicillium species are spore-forming environmental moulds, which may cause spoilage of foods, allergic reactions in humans or even invasive infections in immunocompromised patients (Lyratzopulos et al. 2002, Ndagano et al. 2011). In addition, A. fumigatus is the most common cause of pulmonary aspergillosis. Trichophyton species are dermatophytic fungi (Nir-Paz et al. 2003).

Interestingly, Hamid et al. (1997) showed that the amount of HICA production by Peptostreptococcus anaerobicus is increased in the presence of metronidazole. HICA is also one part of pleofungin molecules, which are new depsipeptide antifungal antibiotics (Aoyagi et al. 2007). The amphipathic and hydrophobic nature of the molecule may have a role in the antimicrobial mode of action of HICA (Chen et al. 2007). The antimicrobial spectrum and potency of HICA for clinical use as an antimicrobial agent has not been defined earlier.

### 2.1.3 Other uses and biocompatibility

Commensal bacteria, especially Lactobacillus species are widely studied and used as probiotics for the treatment of unbalanced microflora in oral, intestinal, genitourinal, and burn wound infections (Lombardo 2008, Barrons & Tassone 2008, Peral et al. 2009, Hasslöf et al. 2010). Lactobacillus species are also frequently
utilized in the food industry as starter cultures for ripening foodstuffs by fermentation and for prevention of spoiling. HICA is expressed in bovine whole milk (Ehling & Reddy 2014), fermented dairy products, cheeses (Yvon & Rijnen 2001, Broadbent et al. 2004, Smit et al. 2005), and wines (Van Wyk et al. 1967). In contrast, in Swiss cheese Propionibacterium freudenreichii did not show either production of HICA or conversion of it to KICA (Thierry et al. 2002). The overexpression of HicDH may lead to decrease in aroma or to delayed flavour formation in cheese (Broadbent et al. 2004). As a non-nitrogen substitute in nutrition, HICA increases weight and muscle mass in chicks and rats (Boebel & Baker 1982) and it improves muscle recovery after immobilisation in rats (Lang et al. 2013). In humans, HICA is used for speeding up muscle recovery after exercise with a 1500 mg daily dose (Mero et al. 2010). In addition, HICA reduces C. albicans biofilm-induced inflammatory responses in murine cells (Nieminen et al. 2014b).

The European Food Safety Authority (EFSA; 2012) has approved HICA as a flavouring substance in nutrition. The maximum survey-delivered daily intake (MSDI) is 0.0012 μg/capita/day and theoretical maximum daily intake (mTAMDI) is 3800 μg/capita/day. The dose of the threshold of concern is 1800 μg/capita/day. Since HICA can be metabolised by human tissues and is found naturally in foods it is well tolerated at least in the doses recommended by EFSA.

2.2 Antimicrobials

2.2.1 Biocides

Antiseptics, disinfectants, preservatives, and sterilants belong to the group of topical antimicrobial agents called biocides. They have an important role in the prevention of general out-patient infections and nosocomial infections. They are also used for the treatment of superficial infections or for prevention of the growth of foodborne pathogens. Topical antimicrobials are used superficially on living tissues to prevent bacteremia and further generalised sepsis. In this context, they are called antiseptics. On inanimate surfaces topical antimicrobials are used to prevent infection and spreading of infection, whilst they are called disinfectants. The general aim of topical antimicrobials is to prevent bacterial growth and kill bacteria. In practice, subcidal concentrations or insufficient exposure time in contact with target organisms may lead to inhibition of microbial growth to a lower
extent. Preservatives prevent deterioration of food and medical products. Sterilants are used in medical devices for bacterial killing.

Antimicrobial mechanisms of different biocides are not well described in the literature as the mode of action of different antibiotics and antifungals (Russell 2002, Sheldon 2005). Interaction between biocide and microorganism is needed for antimicrobial activity. This may occur with one to three levels: in outer cellular components, cytoplasmic membrane, or cytoplasmic constituents. (Maillard 2002). Interaction with the cell surface allows invasion of chemical agents into intracellular target components. Viability of the microbial cells may also be affected by surface interaction alone. (McDonnell & Russell 1999). It is suggested that the microbicidal effect and prevention of development of microbial resistance is based on multiple targets of biocide on microbial cells at the same time (Sheldon 2005).

### 2.2.2 Antibiotics and antifungals

The main targets of systemic antimicrobials in bacterial cells are cell wall, protein, folic acid, and nucleic acid biosynthesis. Penicillins, cephalosporins, and glycopeptides belong to the group of bacterial cell-wall biosynthesis inhibitors. Tetracyclines, aminoglycosides, and macrolides inhibit bacterial protein biosynthesis. Sulfonamides and trimetoprim derivatives inhibit folic acid synthesis. Peptide, polypeptide, and lipopeptide antibacterials inhibit cell-membrane function or protein biosynthesis. Antifungal azoles belong to the group of ergosterol-synthesis inhibitors and echinocandins to the fungal cell-wall biosynthesis inhibitors. Polyenes inhibit cell-membrane function and nucleic acid synthesis is inhibited by fluoropyrimidine. (Kanehisa et al. 2014). Resistance of most common human pathogens to nearly every antibiotic and synthetic systemic antimicrobials has been reported. Development of new treatment options for infections will assist in tackling this worldwide problem. (World Health Organization 2014).

### 2.2.3 Non-susceptibility to biocides

Permeability of microbial cell-wall structures and membranes is a principle mechanism that dictates the sensitivity and resistance of microbial species to antimicrobial agents and biocides, in general. Prokaryotic bacteria are simpler in structure than eukaryotic organisms. Bacterial cell layers consist of phospholipids. Gram-positive bacteria have a single cytoplasmic membrane and thick
peptidoglycan cell wall. Gram-negative bacteria possess a cytoplasmic membrane and outer membrane with a thin peptidoglycan layer in the periplasmic space (Maillard 2002). Bacteria also have fewer cell organelles than fungi and their metabolism is simpler. The rigid cell wall structure of fungi includes glucan, mannan, and chitin. The fungal cell membrane covers the nucleus of the cell and it continues around the endoplasmic reticulum and cell organelles such as mitochondria and ribosomes (Arana et al. 2009). The cell wall structure of *Aspergillus* species is highly hydrophobic in comparison to that of bacteria and yeasts. The ability of microbes to adhere, colonize, and invade into tissues; and to produce capsules, endotoxins, and exotoxins, may increase virulence (Peterson 1996). Hyphal formation and filamentation of dimorphic fungal species is associated with virulence and biofilm formation (Shapiro et al. 2011).

According to the current literature, non-susceptibility to biocides can be divided into intrinsic and acquired mechanisms. An intrinsic mechanism is regulated by the microbial innate genome. It may be expressed by impermeability of membranes, biofilm formation, efflux systems, and enzymatic degradation. Gram-positive bacteria are more susceptible to biocides than gram-negative bacteria, mycobacteria, or spores of spore-forming organisms, due to a more permeable cell wall. Hydrophilic porin channels in the outer membrane of gram-negative bacteria prevent and regulate the transport of hydrophilic substances (Denyer & Maillard 2002). Similarly, lipopolysaccharide (LPS) of the bacterial outer membrane prevents permeability (McDonnell & Russell 1999). In some cases, the same efflux pump systems may be responsible for antibiotic resistance and non-susceptibility to biocides (Moken et al. 1997, McMurry et al. 1998, Chuanchuen et al. 2001). In bacterial and fungal biofilms, non-susceptibility to biocides may depend on altered growth rate, adherence of the biocide to biofilms, and inactivation of enzymatic degradation of biocide (Gilbert & McBain 2003, Sheldon 2005, Soto 2013).

Acquired non-susceptibility may be a consequence of mutation or overexpression of the target, plasmid-regulated efflux, or inactivation by enzymes (McDonnell & Russell 1999, Soto 2013). Surface hydrophobicity, dictated by the fatty acid and protein structure of the outer membrane, has an impact on acquired non-susceptibility (Maillard 2002). In staphylococci, plasmid-mediated non-susceptibility for cationic biocides, chlorhexidine digluconate, and quaternary ammonium compounds, was observed (Russell 2000, Poole 2002). In addition, multi-drug efflux-pump coding genes have been found in multidrug-resistant plasmids of *S. aureus* isolates (Costa et al. 2013). The intrinsic and acquired
mechanisms of antifungal resistance are well described, but the fungal non-susceptibility to biocides is not known precisely (Vandeputte et al. 2012).

Resistance of fungal biofilms has been reported for nearly all presently-used antifungals, except for echinocandins and lipid formulations of amphotericin B (Chandra et al. 2005, Perlin et al. 2015). Therefore, biocides are one primary part in the control of fungal infections. It is well recognised that the same strategies of adaptation to toxicity of antibiotics and biocides are used by microorganisms (Sheldon 2005). Bacteria expressing the genes encoding antibiotic resistance mechanisms have been detected also in the oral environment from saliva, supra- and subgingival plaque, tongue, and root canals (Moraes et al. 2015), but it does not necessarily mean non-susceptibility of these isolates to topical antiseptics or disinfectants.

Disinfection is a function of the concentration of an agent and time of exposure in given conditions. The presence of organic load, interactions with other chemicals, formulation methods, temperature, dilution, and test method may have an impact on their antimicrobial activity. Increasing contact time and different substitutes may enhance the activity of biocides. (McDonnell & Russell 1999). The role of antimicrobial agents in root canal treatment is to improve mechanical eradication by reducing or killing of microbial cells as irrigants or interappointment medications. Topical antibiotics have provided no benefit over Ca(OH)$_2$ intracanal treatment (Longman et al. 2000). Potential resistance and narrower spectrum of antibiotics makes them unwarranted instead of biocides with broad-spectrum activity (Zehnder 2006). The most generally used antimicrobials, agents with clinical antimicrobial relevance for disinfection and interappointment medication in root canal treatments, and agents chemically in close relation to them are discussed below.

2.3 Endodontic disinfectants

2.3.1 Alcohols

The most widely-used antimicrobial alcohols are ethyl alcohol, isopropyl alcohol, and n-propanol. They have broad-spectrum activity against bacteria and fungi, but they are not sporicidal. Ethanol is mainly used for antisepsis, and isopropanol for disinfection and preservation. (Block 1991) Optimal antimicrobial concentration is 60–90%. Isopropyl alcohol is more lipophilic than ethyl alcohol. Membrane
damage and protein denaturation are their suspected mode of actions that, especially in water solutions, lead to interferences in metabolism and to lysis of microbial cells. Denaturation of dehydrogenases of *E. coli* is reported to support that suggestion. (McDonnell & Russell 1999). Alcohols are used as a diluent in commercial disinfectant products in endodontics.

### 2.3.2 Biguanides

Biguanides are a large group of chemical agents consisting of biguanide molecule (C₇H₁₇N₅). Some have hypoglycemic, antimicrobial (Weinberg 1968), or even antineoplastic activity (Pollak 2012). Chlorhexidine is a well-known member of this group. It is a positively-charged synthetic bisbiguanide, in which two biguanide groups and 4-chlorophenyl rings are attached with a central hexamethylene chain (Greenstein *et al.* 1986). This strong base is soluble and stable in water as a gluconate, acetate, or hydrochloride salt. Their activity is prevented by anionic and non-ionic surface-active agents and reduced by phospholipids and organic matter. (Russell & Day 1993).

In general, a low concentration of chlorhexidine increases the cell-membrane permeability of microbes and may cause leakage of agents with low molecular weight out of the cell, especially potassium and phosphorous. Higher concentration of chlorhexidine leads to cell death by precipitation of the cytoplasmic contents. (Hugo & Longworth 1964). Chlorhexidine has rapid broad-spectrum activity against gram-positive and gram-negative bacteria (Mohammadi & Abbott 2009, Haapasalo *et al.* 2010, Gomes *et al.* 2013, Kulik *et al.* 2015). It is also highly active against *Candida* spp. (Salim *et al.* 2013). As a lipophilic and hydrophobic agent, chlorhexidine targets microbial cell-membrane phospholipids and lipopolysaccharides (Athanassiadis *et al.* 2007, Cheung *et al.* 2012). Microbial cell-membrane phosphate groups are negatively charged and the chlorhexidine molecule interacts with them (Hugo & Longworth 1964). Rapid permabilisation of the cell membrane is followed by its disruption (Athanassiadis *et al.* 2007). The inner membrane of gram-negative bacteria is the main target for further interactions in cytosol (Russell & Furr 1987, McDonnell & Russell 1999). Fast intake into the cell is reported in *E. coli* (Hugo & Longworth 1966, Fitzgerald *et al.* 1989, Cheung *et al.* 2012), *S. aureus* (Hugo & Longworth 1966), and *Pseudomonas aeruginosa* cells (Fitzgerald *et al.* 1989). Hiom *et al.* (1992) showed it also enters yeast cells of *C. albicans*, *C. glabrata*, and *Saccharomyces cerevisiae*. Cheung and colleagues (2012) showed that potential sites of chlorhexidine activity were in different parts
on the cell wall of gram-positive and gram-negative bacteria. They also found up-regulation and down-regulation of several proteins of *Bacillus subtilis* and *E. coli*, but these proteins were totally different between these species. Chlorhexidine also attenuates inflammatory responses caused by lipoteichoic acid (LTA) of *E. faecalis* in murine macrophages, in which tumor necrosis factor-α receptor activity and toll-like receptor-2 stimulation are reduced (Lee *et al.* 2009). Development of microbial resistance to chlorhexidine during long-time use might be more evident than in short-time use (Kulik *et al.* 2015).

In endodontics, chlorhexidine is used mainly as a root canal irrigant, but it is recommended in the treatment of Ca(OH)₂-resistant strains as an interappointment medication. The activity of chlorhexidine is pH-dependent and can be decreased with organic matter (Russell & Day 1993, Haapasalo *et al.* 2000, Oliveira de *et al.* 2010). Chlorhexidine forms a precipitate with NaOCl or EDTA, which may have a harmful influence on the activity of antimicrobials and on the dissolving capacity of organic and inorganic tissues of the two latter ones (Rossi-Fedele *et al.* 2012). Chlorhexidine has no tissue-dissolution capacity (Naenni *et al.* 2004, Okino *et al.* 2004). The substantivity of chlorhexidine to dentine may be one useful property of chlorhexidine in comparison to present root canal medicaments. It can be adsorbed into and released from hydroxyapatite of dentine. (Carrilho *et al.* 2010). This may lengthen the antimicrobial activity (Souza de *et al.* 2011) and reduce bacterial colonisation in dentine tubules (Basrani *et al.* 2003).

In clinical medicine, allergic reactions such as contact dermatitis, immediate type of hypersensitivity, and even anaphylaxis, caused by chlorhexidine have been diagnosed (Calogiuri *et al.* 2013, Nakonechna *et al.* 2014). Increased risk for contact dermatitis may be a dose-dependent consequence of chlorhexidine use in hand disinfectants (Nagendran *et al.* 2009). The risk for anaphylaxis could be higher with mucosal application (Okano *et al.* 1989). Allergic reactions may be related to the exposure of chlorhexidine in former surgery operations or in other invasive procedures (Garvey *et al.* 2001, Aalto-Korte & Mäkinen-Kiljunen 2006). Even though, the probability of strict contact of disinfectants to living tissues and induction of new allergic responses in root canal treatment is low; patients with a history of mild reactions to chlorhexidine should be recognized to prevent severe reactions later.
2.3.3 Halogen-releasing agents

Sodium hypochloride (NaOCl) is a bactericidal and fungicidal agent. NaOCl dissolves to hypochlorite ions and hypochlorous acid in water. Temperature and pH have an impact on its antimicrobial activity. Undissolved hypochlorous acid is more active than dissolved hypochlorite. Hypochlorous acid dominates nearby neutral and slightly acidic conditions. (McKenna & Davies 1988, Barrette et al. 1989). The main mode of action of NaOCl is protein denaturation. Chlorine-releasing agents may cause chlorinated derivatives of nucleotide bases in bacterial DNA (Dennis et al. 1979). Disruption in membrane-associated activity by hypochlorous acid is reported, especially in oxidative phosphorylation (Barrette et al. 1989). DNA synthesis is disrupted more than protein synthesis or bacterial membrane (McKenna & Davies 1988).

Because of its antimicrobial activity, tissue-dissolution capacity, and activity against bacterial biofilms it is considered to be the most important agent in root canal irrigation (Gutiérrez et al. 1990, Haapasalo et al. 2010, 2014). NaOCl rapidly loses its activity due to its reactivity. Therefore, thorough application is needed and it cannot be used as an interappointment medication. Inactivation of NaOCl has also been shown by dentine (Haapasalo et al. 2000) and by serum albumin, which may be present in inflammatory exudate (Pappen et al. 2010). High concentrations of NaOCl with long application time may weaken the strength of dentine (Sim et al. 2001, Marending et al. 2007). It is noteworthy that tissue irritation and cell toxicity of NaOCl is a major caution, which should be paid attention to in clinical use (Spångberg et al. 1973, Hülsmann & Hahn 2000).

Iodine is less reactive than chlorine, but its broad-spectrum antimicrobial action occurs rapidly, too. After invasion through cell membrane, iodine attacks proteins such as cysteine and methionine, as well as nucleotides and fatty acids resulting in the death of microbial cells. Iodine molecule (I₂) plays the main role in antimicrobial activity in aqueous solutions (Gottardi 1991). Iodine potassium iodide (IPI) has no capacity to dissolve tissues. In a case of persistent root canal infection, a final rinsing with IPI may extend the antimicrobial effect of conventional irrigation regimen (Molander et al. 1999, Kvist et al. 2004, Haapasalo et al. 2010). In addition, there are some promising results of its ability to disinfect dentine tubules (Safavi et al. 1990), which may also increase the activity of Ca(OH)₂ as an intracanal dressing (Cwikla et al. 2005). Toxic effects caused by overdose oral administration of iodine compounds and reactions to iodinated contrast agents in imaging techniques are better described in the literature than
potential allergies and staining effects of iodine compounds in topical use (Backer & Hollowell 2000, Pasternak & Williamson 2012).

### 2.3.4 Hydroxyl ion-releasing agents

Hydrogen peroxide is a renowned biocide with a rapid release of hydroxyl ions, which is in wide use in antisepsis and disinfection. It is a fast-acting, broad-spectrum, antimicrobial agent for bacteria and yeasts, which denatures proteins, lipids, and DNA. Free hydroxyl radicals oxidize thiol groups of proteins and enzymes. DNA strands are broken by hydrogen peroxide. (McDonnell & Russell 1999). Hydrogen peroxide decreases the microhardness of dentine (Lewinstein et al. 1994). The antimicrobial activity of hydrogen peroxide has not been very good in comparison to more commonly used irrigants in endodontics (Viljaykumar et al. 2010).

Ca(OH)₂ paste is well-known due to its slow release of hydroxyl ions. This chemical property is utilized in dentistry and environmental chemistry. The uses in dentistry are related to its antimicrobial activity and its ability to stimulate mineralisation (Foreman & Barnes 1990). Ca(OH)₂ is slightly soluble in water. The physical constant of hydrolysed ions in aqueous saturated solution of Ca(OH)₂ is 1.65 mg/mL at 20°C and at body temperature this is a little lower (1.45 mg/mL; Dean 1973). There is also a marginal decrease in pH, if the temperature is increased (Bates et al. 1956). The antimicrobial activity of Ca(OH)₂ is attributed to high alkalinity (pH 12.5) of saturated solution. Undersaturation of solution leads to reduction of hydroxyl-ion concentration and pH. The targets of Ca(OH)₂ are in the bacterial cytoplasmic membrane, protein denaturation, and possibly in DNA (Halliwell 1987, Imlay & Linn 1988, Siqueira & Lopes 1999, Estrela & Holland 2003). It has activity on the outer membrane antigen, bacterial lipopolysaccharide (LPS) of gram-negative bacteria (Buck et al. 2001, Silva et al. 2002), and on E. faecalis lipoteichoic acid (LTA). Deacylation of LTA leads to attenuation of inflammatory reaction in apical periodontitis. (Baik et al. 2011).

Byström et al. (1985) showed that Ca(OH)₂ was more active in situ than camphorated phenol (CP) or camphorated paramonochlorophenol (CMCP) during a month-long incubation. Sjögren et al. (1991) showed its antimicrobial effect during seven-days of medication in vivo. Nowadays, Ca(OH)₂ paste is the most widely used interappointment medication in endodontics (Law & Messer 2004). Ca(OH)₂ has the ability to dissolve tissues (Hasselgren et al. 1988), which enhances tissue dissolution effect of NaOCl after appointment (Türkün & Cengiz 1997).

2.4 Root canal infections

Apical periodontitis (AP) seems to be more general in prevalence than severe forms of periodontitis (Pilot & Miyazaki 1991, Suominen-Taipale \textit{et al.} 2004). In Nordic countries, the prevalence of AP in adults is over 40\% (Kirkevang \textit{et al.} 2012, Haikola \textit{et al.} 2013, Huumonen & Ørstavik 2013). In the Finnish adult population, root canal treatment was carried out in 7\% of all teeth and it was more general in women than in men (Huumonen \textit{et al.} 2012). In a ten-year follow-up study of root-filled teeth, 13\% were extracted, 12\% were revised, and 42\% had AP (Kirkevang \textit{et al.} 2014). The infection after dental trauma is a common reason for the need of root canal treatment in children (Humphrey \textit{et al.} 2003, Bakland 2012). General changes in population age-distribution may lead to increase in prevalence of periapical lesions of elderly dentate people (Ainamo \textit{et al.} 1994, Haikola \textit{et al.} 2013). Immunocompromised patients have a higher risk for generalized infections also from dental origin. Two thirds of odontogenic maxillofacial abscesses requiring hospital care are from pulpal origin. (Grönholm \textit{et al.} 2013).

2.4.1 Etiology and pathology

AP is a root canal microbial infection-induced inflammatory reaction in the periapical tissues (Kakehashi \textit{et al.} 1965, Sundqvist 1976). Already in 1894, Miller showed the presence of bacteria in necrotic pulps. However, much later Kakehashi
et al. (1965) demonstrated that microbial organisms are causative factors for AP. Other studies have later confirmed that necrotic pulp without microbial infection does not induce periapical inflammation (Sundqvist 1976, Möller et al. 1981). According to present knowledge, microorganisms are commonly arranged as biofilms in human root canals during the disease (Nair 2004, Svensäter & Bergenholz 2004, Ricucci & Siqueira 2010, Siqueira & Rôças 2014). AP is an inflammation reaction induced by intraradicular infection, but occasionally the infection may spread to extraradicular tissues. Sunde and colleagues (2003) showed bacteria in over 50% of periapical lesions of asymptomatic root-filled teeth that required apical surgery by fluorescence in situ hybridization. Dental caries, fractures, and leakages in coronal or root fillings are the most common routes of infection, from which any oral organism may potentially contaminate pulpal tissues. Additional root canals, malformations in dental and dentine structures, active root resorption and occlusal trauma increase the risk for pulpal infection. Microbial colonization from root surface via cemento-deficient areas and hematogenous routes have also been suggested (Stashenko et al. 1998, Nair et al. 2004).

**Microbes**

Bacteria are considered to be the main causative organisms of AP, even though viruses, archaea, and fungi are also found (Siqueira & Rôças 2014). About one third of 500 bacterial species detected in endodontic infections are culturable. One quarter of them have been detected with both conventional culture methods and with molecular biology methods such as polymerase chain reaction (PCR), checkerboard, broad-range PCR, cloning, sequencing, terminal-restriction fragment length polymorphism, reverse-capture checkerboard, real-time PCR, or microarray analyses. (Siqueira & Rôças 2009). Next-generation DNA sequencing (NGS) techniques, such as pyrosequencing, have already shown that the divergence of microbial species in endodontic infections is far wider than was previously thought and it will broaden our knowledge further in the future (Siqueira & Rôças 2014). Both culture and molecular methods are needed for comprehensive analyses. The organisms distinguished by culture methods are living organisms, and with molecular methods living organisms, and lifeless genomes can be detected.

Growth conditions, especially availability of nutrients, oxygen, and pH in root canal affect the selection of the colonising microbial species and their position in root canal. Therefore, obligately anaerobic species dominate in primary infection and the flora is modified to facultatively aerobic species in secondary infection or
post-treatment disease. (Sundqvist & Fidgor 2003, Peciuliene et al. 2008). Nutritional environment, structure of dentine and bacterial adhesion may influence bacterial invasion to dentinal tubules. Gram-positive streptococcal, enterococcal, and Actinomyces species are able to invade into human root dentinal tubules, while gram-negative species are less frequently recovered (Love & Jenkinson 2002). Dentine tubules invasion of C. albicans is mainly superficial. Some single cells were reported throughout the dentine specimens with approximately 2 mm of thickness ex vivo (Waltimo et al. 2000). In caries lesions, this hardly exists in vivo (Maijala et al. 2007).

**Host response**

Periapical inflammation induced by microbial biofilms in the root canal resembles the immunological reactions in periodontal infection. Initial infiltration of immunocompetent cells and alveolar bone destruction may begin before pulpal necrosis (Kawashima et al. 1996). Dental pulp is prepared for antigenic challenges via dentine tubules. Innate immune reactions mediate the initial antigenic challenges, which include inflammatory-cell infiltration (neutrophils, macrophages, and dendritic cells), and synthesis of inflammatory mediators. Thereafter, mainly T- and B-lymphocyte-mediated adaptive immune reactions are initiated for more specific responses to certain antigens. Inflammatory reactions with restricted blood circulation and decrease in environmental compliance lead to irreversible destruction of pulp tissue. (Kawashima & Suda 2008). Increased levels of cytokines have been detected from inflammatory exudate in periapical lesions (Matsuo et al. 1994) and from fibroblasts in inflammed pulp (Barkhordar et al. 2002, Silva et al. 2009). Bacterial lipopolysaccharide (LPS) induces inflammatory responses, which lead to function of polymorphonuclear (PMN) cells (Hou et al. 2000, Ubagai et al. 2015). In primary infections, the microflora is potentially composed of anaerobic gram-negative rods and LPS-induced reactions may be expressed. Interestingly, Smith et al. (2012) showed that extracellular matrix extracts of dentine and pulp have antimicrobial properties.

Remodelling of bone is a balance stage between resorption caused by osteoclasts and formation by osteoblasts. In pathologic states, bone resorption is increased in comparison to bone formation. Regulation mechanisms of this phenomenon are multidimensional. Briefly, bone destruction is mediated and caused by osteoclasts, which are macrophage-derived multinuclear cells. Osteoblastic cytokines, such as macrophage-colony stimulating factor and receptor activator of
NF-κB ligand (RANKL) increase osteoclast formation from osteoclast precursor cells. T- and B-lymphocytes also regulate osteoclast formation by expressing RANKL. Osteoclasts are able to secrete hydrochloric acid and proteases, such as cathepsin K, to dissolve matrix and mineral constituents of bone, which occurs beneath a ruffled part of the osteoclastic basal membrane. (Teitelbaum 2000). Root cementum and dentine may be resorbed by odontoclasts, which have histochemical and structural similarities with osteoclasts (Sahara et al. 1994).

**Biofilms**

Microbes in biofilms are far more resistant to antimicrobials and host response reactions in comparison to planktonic cells (Ceri et al. 1999, Hajo et al. 2009, Lang et al. 2012, Wang et al. 2012). Microbial adherence to a surface is a precondition for biofilm formation. In a mature biofilm, several layers of microbial cells are surrounded by extracellular polymeric substance (EPS), which contains mainly extracellular polysaccharides and proteins. It takes part in aggregation and adhesion of the cells. EPS plays a role in microbial biofilm formation, in protection of microbial cells from environmental impulses, and it prevents diffusion of antimicrobials. (Vu et al. 2009). In dental biofilms, growth-dependent killing may be also reduced, because of slower microbial growth in them. In addition, drug-inactivating enzymes may increase microbial resistance in biofilms. (Hajo et al. 2009). Resistance mechanisms in biofilms may also be attributed to nutritional conditions and adaptation to resistant phenotype (Kishen et al. 2010).

Stojicic et al. (2013) showed that resistance of microbial cells to disinfectants increases in experimental multispecies biofilms on collagen-coated hydroxyapatite disks with time. Intraradicular biofilm formation was reported in 74–80% of untreated and treated teeth with AP (Ricucci & Siqueira 2010). Chávez de Paz (2007) showed that species of bacteria usually living in acidic environments such as Streptococcus spp., Lactobacillus spp., and F. nucleatum were able to adapt and stay viable in up to pH 10.5 biofilms for 4 h. The biofilm community is well protected from the defence system of the host (Lang et al. 2012). Especially, biofilms may be detected on root canal walls, whereas some microbial cells may disperse into dentinal tubules.
2.4.2 Microbial species

In primary endodontic infections

In primary infections, commonly isolated organisms are anaerobic gram-negative rods (*Fusobacterium nucleatum*, *Campylobacter rectus*, *Tannerella forsythia*, *Prevotella* and *Porphyromonas* spp.), and spirochetes (*Treponema* spp.), anaerobic and facultative gram-positive cocci and rods (*Peptostreptococcus* spp., *Eubacterium* spp., *Propionibacterium* spp., *Actinomyces* spp., streptococci, lactobacilli) (Sundqvist 1994, Sousa de et al. 2003, Gomes et al. 2004, Rôças & Siqueira 2008, Sakamoto et al. 2009, Siqueira & Rôças 2014). Bacterial species altogether from nine different phyla (*Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes, Synergistetes, SR1, and TM7*) have been detected by culture or molecular methods. Bacterial species from nine other phyla have been detected by pyrosequencing techniques, but they may be rare species. There is clearly more divergence in the microflora of symptomatic versus asymptomatic infections, which may be related to higher virulence and pathogenicity (Santos et al. 2011, Siqueira & Rôças 2014).

In post-treatment endodontic infections

Facultatively anaerobic gram-positive cocci and rods dominate the microflora in post-treatment disease such as *Streptococcus, Enterococcus, Peptostreptococcus*, and *Actinomyces* species. The proportion of *Lactobacillus* species and anaerobic gram-negative rods is lower than in primary infection. In addition, enteric rods have often been isolated from root canals in post-treatment AP. (Molander et al. 1998, Peciuliene et al. 2001, 2008, Chávez de Paz et al. 2003, 2004, Pinheiro et al. 2003, Rôças & Siqueira 2008, Siqueira & Rôças 2014). Fungal species have also been relatively often (in 5–20% of cases) isolated from AP, especially in persisting infections. The most commonly isolated species is the dimorphic fungus *C. albicans*. *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, and *C. krusei* are isolated to a lesser extent (Waltimo et al. 1997, 2001, 2003, Sinha et al. 2013, Kumar et al. 2015).


**Enterococcus faecalis**

*E. faecalis* is widely used as a test organism in endodontic studies during the last two decades. A PubMed search (October 15th, 2015) yielded 535 studies with the terms “*E. faecalis* and root canal”. The extent of research into this topic is based on the fact that *E. faecalis* has been the most-often detected organism from post-treatment infections (Portenier *et al.* 2003, Stuart *et al.* 2006, Zehnder & Guggenheim 2009, Haapasalo *et al.* 2011, Lew *et al.* 2015, Zhang *et al.* 2015). Some molecular method studies support the major prevalence of *E. faecalis* in persisting AP (Rôças *et al.* 2004, Sedgley *et al.* 2006), while some studies do not (Rolph *et al.* 2001).

*E. faecalis* is a gram-positive facultatively aerobic cocci. It is a saprophytic commensal organism (Toledo-Arana *et al.* 2001), which belongs to the normal intestinal flora and may also cause endocarditis, urinary tract, and skin wound infections. *E. faecalis* is rarely found in the oral cavity but may originate from food items (Razavi *et al.* 2007). It has intrinsic resistance to antibiotics such as β-lactams and clindamycin; and may have acquired resistance to several antibiotics such as tetracyclines, macrolides, glycopeptides, chloramphicol, and aminoglycosides (Portenier *et al.* 2003, Moraes *et al.* 2015). *E. faecalis* is able to invade into human dentinal tubules (Safavi *et al.* 1990, Love & Jenkinson 2002) and is highly resistant to alkaline stress (Flauhaut *et al.* 1997, Weckwerth *et al.* 2013). The ability of *E. faecalis* to adhere to dentine is reported to improve the resistance to Ca(OH)$_2$ (Brändle *et al.* 2008). Proton pump function is necessary for the survival of *E. faecalis* in high alkalinity (Evans *et al.* 2002).

Especially in post-treatment disease, LTA of *E. faecalis* may increase inflammatory responses (Lee *et al.* 2009, Baik *et al.* 2011). Differentiation of osteoclasts may be reduced by *E. faecalis*, while macrophages sustain their capacity to phagocytose and to induce secretion of proinflammatory cytokine and chemokine (Park *et al.* 2015). However, *E. faecalis* may be present in the infected root canals also without visual AP (Kaufman *et al.* 2005). In the experimental conditions, Ozdemir *et al.* (2010) showed biofilm formation of *E. faecalis* in the root canals of human teeth after 24 h.

**Microbial species in extraradicular infections**

Bacterial *Actinomyces* species and *Propionibacterium propionicum* are causative organisms of independent extraradicular actinomycosis, but its role in endodontic
failures is controversial (Nair 2004, Siqueira & Roças 2014). Fungal *Aspergillus* species are common colonisers of the upper and lower airways and they belong to group of the most important pathogens in fungal infections together with yeasts. They are spore forming and dimorphic moulds. Filamentation of *A. fumigatus* is associated with pathogenesis. Systemic infections typically arise from colonisation. Most *Aspergillus* infections are related to immunodeficiency. (Kousha et al. 2011). The role of dental root-canal fillings and the over-fillings have been discussed in connection with *Aspergillus* sinusitis (Legent et al. 1989). Surgical treatment is needed in extraradicular dental infections.

### 2.4.3 Treatment

Root canal treatment is the treatment of choice in irreversible pulpitis, pulpal necrosis, or AP. The overall aim of root canal treatment is to eradicate root canal infection and to prevent or to heal AP. Three different parts of the treatment protocol affects the prognosis of the disease: mechanical preparation, chemical disinfection, and prevention of infection reactivation. This work is focused on the second one. Destruction of biofilms by mechanical preparation is a cornerstone of the treatment of biofilm infections including endodontic infections (Byström & Sundqvist 1981, Dalton et al. 1998). Mechanical preparation includes hand and rotary instrumentation and hydrodynamic effects during irrigation. Peters et al. (2001) showed that even 35% of root canal system may be left uninstrumented after preparation with hand or rotary instrument. Therefore, chemical agents are needed for improvement of antimicrobial effect by irrigation and interappointment medication.

The aim of root canal medication is to kill bacteria that either grow planktonically or are arranged in biofilms. Subcidal concentration of an antimicrobial agent in contact with microbes or insufficient exposure time can lead to bacteriostatic effect, which may assist in infection control in favourable conditions, too. NaOCl is the most active root canal irrigant against microbial biofilms: the higher the concentration or the longer the contact time, the better result is achieved (Zehnder 2006, Haapasalo & Shen 2012). Ever since Byström and collegues (1985) showed better activity of Ca(OH)$_2$ in comparison to CP or CMCP, and Safavi et al. (1985) and Sjögren et al. (1991) showed its antimicrobial activity *in vivo* Ca(OH)$_2$ has become the most generally used interappointment medication in endodontics (Law & Messer 2004). One explanation for the superior activity of Ca(OH)$_2$ may be that in the group of Ca(OH)$_2$-treated teeth, Byström et
al. (1985) used 5% NaOCl in irrigation of nearly half of the subjects (n=15 of 35). On the contrary, all subjects of the group of CMCP or CP-treated teeth were irrigated only with 0.5% of NaOCl. Sjögren et al. (1991) used 0.5% NaOCl for all subjects and they confirmed these results by 18 subjects with Ca(OH)₂ medication for seven days. Nine of them were bacteria free after the irrigation before interappointment medication and all of them were disinfected after the medication. It is noteworthy that the results of the clinical microbiological studies show the combined effect of disinfection and interappointment medication, although these are not clearly reported separately. Safavi et al. (1985) observed the effect of interappointment medication after disinfection. All initial samples, which were taken after irrigation from root canals were free of bacterial growth, but after seven days of medication with Ca(OH)₂, 77% of the subjects were bacterial free. The same was evident for 66% of teeth with IPI intracanal medication. Despite conflicting evidence for antimicrobial activity of Ca(OH)₂ in root canal treatments both in vitro (Ørstavik et al. 1991, Reit et al. 1999, Kim & Kim 2014) and in vivo (Table 1), new approaches have not displaced it. The results of the clinical outcomes analysed by molecular methods (Souza de et al. 2005, Siqueira et al. 2007, Rôças et al. 2011) and histobacteriological methods (Vera et al. 2012) are in line with studies analysed by conventional culture methods shown in the underlying comparison.

E. faecalis, which was described in the previous section of this thesis, and C. albicans are especially non-susceptible to Ca(OH)₂ (Waltimo et al. 1999, Portenier et al. 2003, Delgado et al. 2013, Siqueira & Rôças 2014). Alkaline-resistant strains are found mostly in post-treatment infections, but also sometimes in primary infections (Lew et al. 2015). In addition, interactions with dentine, necrotic soft tissue, or serum albumin may lead to inactivation of root canal disinfectants (Haapasalo et al. 2000, 2007, Portenier et al. 2001, 2002, 2006, Oliveira de et al. 2010). More active irrigation and disinfection with chlorhexidine, NaOCl, and IPI, or short chlorhexidine interappointment medication with broader spectrum activity against Ca(OH)₂-tolerant species are recommended in the treatment of these infections (Waltimo et al. 1999, Haapasalo & Shen 2012). However, their potential tissue toxicity or allergic reactions cannot be disregarded (Aalto-Korte & Mäkinen-Kiljunen 2006, Pasternak & Williamson 2012, Guleri et al. 2012, Gomes et al. 2013), and the evidence of their clinical efficacy is lacking.
Table 1. Antimicrobial activity of disinfective irrigation and Ca(OH)\(_2\) medication detected after 7 days up to 2 months in clinical conditions. A specific detection time was not described in one study.

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Analyses</th>
<th>N</th>
<th>N/group</th>
<th>7d</th>
<th>14d</th>
<th>1–2mon</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td>Byström et al.</td>
<td>Culture</td>
<td>65</td>
<td>30–35</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1985</td>
<td>Safavi et al.</td>
<td>Culture</td>
<td>1030</td>
<td>173–517</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1991</td>
<td>Sjögren et al.</td>
<td>Culture</td>
<td>30</td>
<td>12–18</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1997</td>
<td>Barbosa et al.</td>
<td>Culture</td>
<td>120</td>
<td>36–45</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td>Molander et al.</td>
<td>Culture</td>
<td>50</td>
<td>22</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>Shuping et al.</td>
<td>Culture</td>
<td>47</td>
<td>5–26</td>
<td>*</td>
<td>(7–200d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Peters et al.</td>
<td>Culture</td>
<td>43</td>
<td>21</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Tang et al.</td>
<td>Molecular</td>
<td>31</td>
<td>6–25</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Zerella et al.</td>
<td>Culture</td>
<td>40</td>
<td>20</td>
<td>+</td>
<td>(7–10d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Souza de et al.</td>
<td>Molecular</td>
<td>12</td>
<td>12</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>Chu et al.</td>
<td>Culture</td>
<td>88</td>
<td>12–19</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Manzur et al.</td>
<td>Culture</td>
<td>33</td>
<td>11</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Vianna et al.</td>
<td>Culture</td>
<td>24</td>
<td>8</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Siqueira et al.</td>
<td>Molecular</td>
<td>11</td>
<td>11</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Rôças et al.</td>
<td>Molecular</td>
<td>24</td>
<td>12</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Vera et al.</td>
<td>Histological</td>
<td>13</td>
<td>6–7</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>Sinha et al.</td>
<td>Culture</td>
<td>90</td>
<td>30</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

++: good activity (90% of the subjects free of bacterial growth), +: limited activity (50–90%), -: no activity (<50%), ND: not described.

Finally, at the end of and after root canal treatment, reactivation of infection is mechanically prevented by tight root canal filling and coronal filling. The pathways between the root canal and periapical tissues, and to the oral cavity should be sealed. Nutrition of potentially present microbial cells has to be prevented. Conventionally-used gutta-percha filling material lacks antimicrobial activity. Some epoxy resin-based, polymethylacrylate resin-based and calcium silicate-based sealers may have antimicrobial properties in some experimental conditions (Ustun et al. 2013), but the results are controversial (Heyder et al. 2013). In addition to good sealing ability, mineral trioxide aggregate (MTA), which includes calcium, silica, and bismuth, has antimicrobial activity (Estrela et al. 2000, Parirokh & Torabinejad 2010).
Single-visit treatment

The strategy of one-visit treatment is based on the fact that mechanical preparation and disinfection during irrigation at the appointment is sufficient and further operation would not improve the quality of root canal treatment. In non-infected diseases, such as in pulpitis or sterile pulpal necrosis, this is accurate (Trope & Bergenholtz 2002), but in root canal infections the evidence is lacking. Although, it has been shown, that the elimination of microbes from the root canal system in multiple-visit treatment with Ca(OH)$_2$ is not significantly better than single-visit treatment \textit{ex vivo} (Vivacqua-Gomes \textit{et al.} 2005) or \textit{in vivo} (Weiger \textit{et al.} 2000, Shuping \textit{et al.} 2000, Kvist \textit{et al.} 2004, Molander \textit{et al.} 2007, Penesis \textit{et al.} 2008, Paredes-Vieyra & Enriquez 2012, Vera \textit{et al.} 2012). However, Byström & Sundqvist (1983) showed before the first clinical studies of Ca(OH)$_2$ that with 0.5% NaOCl irrigation, and with saline as an interappointment root-canal dressing, bacterial growth was still detected in 50% of the root canals at second appointment. Sjögren and collegues (1987) also confirmed this after chemo-mechanical preparation. Vera \textit{et al.} (2012) showed lower amounts of bacteria in different parts of root canal system in histological analyses, including dentine tubules after interappointment medication versus single-visit treatment. Therefore, interappointment medication appears to be useful for the reduction of bacterial growth in infected root canal system.

Prognosis of the disease

Population studies have shown successful healing in 60–74% of the treatment of AP and the studies performed in universities have revealed around 90%. (Ray & Trope 1995, Trope & Bergenholtz 2002). Surrounding deep periodontal pockets (Ørstavik \textit{et al.} 2004) and large lesion sizes (Chugal \textit{et al.} 2001) are preconditions that may lead to poorer clinical outcomes of root canal treatment. In a long run, the best treatment outcomes in radiological analyses are associated with good quality of both root canal filling and coronal restoration (Gillen \textit{et al.} 2011, Song \textit{et al.} 2014, Kirkevang \textit{et al.} 2014). According to the literature, major associations with failure in root canal treatment is because of microbial factors (Molander \textit{et al.} 1998, Waltimo \textit{et al.} 2005, Peciuliene \textit{et al.} 2008, Haapasalo \textit{et al.} 2011, Siqueira & Rôças 2014), whereas other factors, such as true cysts and cholesterol crystals may cause the disease far more rarely (Nair 2004). Insufficient microbial elimination from the root canal system may lead to difficult-to-treat persisting infections, in which
enterococci: mainly *E. faecalis*, and enterobacteria and *C. albicans* are often found (Sen et al. 1995, Peciuliene et al. 2001, 2008, Waltimo et al. 2003, Portenier et al. 2003, Kumar et al. 2015). Despite the promising results of the activity of chlorhexidine and sodium hypochlorite *in vitro* the ability to eliminate *E. faecalis* *in vivo* has only been moderate (Estrela et al. 2008).

In clinical follow-up studies, the mean outcome of healed cases in the treatment of primary AP has been 79% (range 46–97%) and in post-treatment disease 76% (range 28–98%) (Friedman 2008). Clinical outcomes can still be improved. Better chemomechanical methods and more active antimicrobials in comparison to currently used ones would assist to resolve this problem. In general, microbial susceptibility to antimicrobials can be determined in minimum inhibitory concentration (MIC) assays. Microbial resistance leads to increases in the minimum inhibitory concentration (MIC) of systemic antimicrobials, which is associated with poorer clinical outcomes and infection complications during treatment and prophylaxis (Pfaller 2012). The concentration as a function of time is the main question for the disinfectant activity (Russell & McDonnell 2000). The higher antimicrobial activity of an agent is without doubt a benefit in the complex microanatomy of root canal system including dentine tubules. Safe antimicrobial agents with new mode of actions are needed for the management of topical infections in root canals.
3 Aims of the study

The aims of the present study were:

1. To evaluate the susceptibility of facultative and strictly anaerobic gram-positive and gram-negative bacterial species to HICA for the evaluation of its antibacterial spectrum.
2. To determine the susceptibility of yeasts and moulds to HICA to evaluate its antifungal spectrum and further observe the influence of HICA on hyphal formation of *C. albicans* and filamentation of *A. fumigatus* cells.
3. To compare the antibacterial activity of HICA to currently-used root canal medicaments and the impact of potential inhibitors, such as dentine, hydroxyapatite and serum albumin, on their activity.
4. To examine the antibacterial activity of HICA in the experimentally-infected root canals of extracted human teeth and to compare the activity to presently-used intracanal interappointment medications in the environment, which mimic clinical conditions.
4 Materials and methods

The main objectives of this project were the antimicrobial properties of DL-HICA in general and its suitability for the treatment of root canal infections. The project consisted of two study entities. In the first two studies, the antimicrobial spectrum of HICA was evaluated, \textit{in vitro} (Study I and II). In the other two studies the suitability of HICA for the treatment of root canal infections was evaluated, \textit{in vitro} (Study III) and \textit{ex vivo} (Study IV). (Table 2).

\textbf{Table 2. Summary of the studies (I–IV) and experiments (a–d).}

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Description of intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>Susceptibility testing of aerobically cultured bacteria</td>
</tr>
<tr>
<td>Ib</td>
<td>Susceptibility testing of anaerobically cultured bacteria</td>
</tr>
<tr>
<td>Ila</td>
<td>Susceptibility testing of \textit{Candida} species</td>
</tr>
<tr>
<td>Ilb</td>
<td>Susceptibility testing of \textit{Aspergillus} species</td>
</tr>
<tr>
<td>IIc</td>
<td>Susceptibility testing of fungi for azoles</td>
</tr>
<tr>
<td>IId</td>
<td>Hyphal formation in \textit{C. albicans} and \textit{A. fumigatus} isolates</td>
</tr>
<tr>
<td>IIIa</td>
<td>Antibacterial activity with inhibitors in nutrient-deficient conditions</td>
</tr>
<tr>
<td>IIIb</td>
<td>Antibacterial activity with inhibitors in nutrient-rich conditions</td>
</tr>
<tr>
<td>IV</td>
<td>Antibacterial activity in the human dental root canals, \textit{ex vivo}</td>
</tr>
</tbody>
</table>

The susceptibility of the isolates of human pathogenic gram-positive and gram-negative bacterial species (n=18) was examined to HICA. Methicillin-resistant \textit{S. aureus} (MRSA) isolate and \textit{E. coli} isolate with extended spectrum beta-lactamase (ESBL) enzyme were included. The susceptibility of \textit{Candida} isolates (n=10) and \textit{Aspergillus} isolates (n=9) to HICA and to azole antifungal agents was determined. (Table 3). The modifications of the standard microdilution methods for susceptibility testing were used (Study Ia, Ib, Ila, Ilb, and Ilc). The impact of HICA on hyphal formation, filamentation, and cell-wall structures of \textit{C. albicans} and \textit{A. fumigatus} isolates was evaluated in germination studies (Study IId).

The antibacterial activity against \textit{E. faecalis} and suitability of HICA for root canal medication was evaluated with potential inhibitors (Study IIIa and IIIb) and in the infected human dental root canals (n=73; Study IV). The Ethics Committee of Northern Ostrobothnia Hospital District approved the use of human teeth (decision number 19/2006). The antimicrobial-inhibitor interactions were tested in nutrient-deficient environment by the dentine powder method (Haapasalo \textit{et al.} 2000) and nutrient-rich environment by microdilution method (Jorgensen \textit{et al.} 2000).
Bacterial invasion into dentine tubules was observed in the *ex vivo* experiment (Study IV).

Table 3. Bacterial and fungal strains, designations, and sources used in the studies (I–IV) and experiments (a–d).

<table>
<thead>
<tr>
<th>Organism and phenotype</th>
<th>Designation</th>
<th>Source</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em>¹</td>
<td>ATCC 25923</td>
<td>NA</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>T-54136</td>
<td>Blood</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em>, MRSA</td>
<td>UKNEQAS 3/2011</td>
<td>NA</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em>¹</td>
<td>ATCC 29212</td>
<td>Urine</td>
<td>Ia, IIb</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>T-75359</td>
<td>Root canal</td>
<td>Ia, IIIa,b, IV</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em>¹</td>
<td>ATCC 13419</td>
<td>Saliva</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>T-75354</td>
<td>Root canal</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Actinomyces israelii</em>²</td>
<td>ATCC 12102</td>
<td>Brain abscess</td>
<td>Ib</td>
</tr>
<tr>
<td><em>Actinomyces israelii</em></td>
<td>T-90969</td>
<td>Gingival sulcus</td>
<td>Ib</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em>²</td>
<td>ATCC 53103</td>
<td>Feces</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Lactobacillus spp.</em></td>
<td>T-75020</td>
<td>Root canal</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Escherichia coli</em>³</td>
<td>ATCC 25922</td>
<td>NA</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>T-54107</td>
<td>Blood</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, ESBL</td>
<td>NCTC 13353</td>
<td>NA</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em>³</td>
<td>ATCC 27853</td>
<td>Blood</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>T-50866</td>
<td>Blood</td>
<td>Ia</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em>³</td>
<td>ATCC 25586</td>
<td>Cervico-facial lesion</td>
<td>Ib</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>PD 788</td>
<td>Gingival sulcus</td>
<td>Ib</td>
</tr>
<tr>
<td><em>Candida albicans</em>³</td>
<td>ATCC 10231</td>
<td>Bronchomycosis</td>
<td>Ila,c,d</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>D 17</td>
<td>Root canal</td>
<td>Ila,c,d</td>
</tr>
<tr>
<td><em>Candida dubliniensis</em></td>
<td>T-98 QC 2000</td>
<td>NA</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>EO 323</td>
<td>Oral</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>ATCC 750</td>
<td>Bronchomycosis</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>D 213</td>
<td>Oral</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td>CCUG 32725</td>
<td>Blood</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td>G 212</td>
<td>Oral</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
<td>ATCC 6258</td>
<td>Sputum</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
<td>D 206 B</td>
<td>Oral</td>
<td>Ila,c</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em>³</td>
<td>MRCM 24290</td>
<td>Sputum</td>
<td>IIb,c,d</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>MRCM 32208</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>MRCM 32221</td>
<td>Sputum</td>
<td>IIb,c,d</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>MRCM 32552</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>ATCC 204304</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>MRCM 23135</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>MRCM 24832</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>MRCM 25730</td>
<td>Sputum</td>
<td>IIb,c</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>MRCM 26158</td>
<td>Bronchial wash</td>
<td>IIb,c</td>
</tr>
</tbody>
</table>

¹Gram-positive cocci (n=7); ²Gram-positive bacilli (n=4); ³Gram-negative bacilli (n=7); ⁴Yeasts (n=10);
⁵Moulds (n=9); NA: not applicable
4.1 Materials

4.1.1 Microbial isolates and strains

Clinical isolates were obtained from the Clinical Microbiology Laboratory Helsinki University Central Hospital HUSLAB (T and PD isolates), the Institute of Dentistry, University of Helsinki (D and G isolates), and the Mycology Reference Centre Manchester (MRCM; UK). The reference strains were obtained from the American Type Culture Collection (ATCC; USA), the Culture Collection of the University of Gothenburg (CCUG; DK), National Collection of Type Cultures (NCTC; UK), and United Kingdom National External Quality Assessment Service (UK NEQAS).

Bacterial isolates had been speciated by colony morphology, microscopy, biochemical methods, or by 16s sequencing. Fungal isolates had been identified by morphological and biochemical conventional methods, and by ITS1 and ITS2 rRNA sequencing (White et al. 2011). They were stored in milk-glycerine at -70°C and cultured twice on agar before the experiments for 48 h, except in anaerobic conditions for seven days. *E. faecalis* isolates (n=2) were chosen for the suitability testings of HICA for endodontic use.

4.1.2 Culture conditions and dilutions

The reagents, agars, culture broths, and their pHs, and potential inhibitors used in the experiments are listed in Table 4. All incubations were performed at 37°C: in 5% CO₂ for facultatively anaerobic bacteria, in anaerobiosis for *Fusobacterium nucleatum* and *Actinomyces israelii*, and in aerobic conditions for fungi. For bacterial susceptibility testing, HICA was diluted from 200 mg/mL aqueous solution into broth (Study Ia, Ib, Ia, IId and IIb). For susceptibility testing of *Aspergillus* species for HICA and fungi for azoles, the test concentrations of antimicrobials were dissolved directly into the broth (Study IIb and IIc). In the dentine powder and root canal infection models antimicrobials were mixed with sterile deionised water (Study IIIa and IV). In the susceptibility testings, commercial chlorhexidine digluconate (CHG) of 2 mg/mL (Corsodyl®, Study Ia and IIb; Curasept®, Study IIa, IIb, IIc and IIIb) were used as positive controls to avoid precipitation and to fulfill the optical requirements of the spectrophotometric analyses. The culture broth was used as a negative control to determine the maximum microbial growth, except in the nutrient-deficient environments where
1.7 mg/mL of peptone water (Study IIIa) or sterile saline (Study IV) served as the negative controls.

Table 4. Final test concentrations (mg/mL) of the reagents used in studies (I–IV) and experiments (a–d) including antimicrobials, comparators, culture media, and potential inhibitors and their pHs.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Manufacturer</th>
<th>pH</th>
<th>mg/mL</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-HICA</td>
<td>TCI Europe, BE</td>
<td>5.2</td>
<td>0.6–36</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18–72</td>
<td>a,b,d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33.3</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36–108</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8</td>
<td>4.5–36</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DL-leucine</td>
<td>TCI Europe, BE</td>
<td>5.2</td>
<td>4.5–36</td>
<td>a,b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18–72</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DL-KICA</td>
<td>TCI Europe, BE</td>
<td>5.2</td>
<td>18–72</td>
<td>a</td>
<td>a,d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fluconazole (mg/L)</td>
<td>Pfizer, UK</td>
<td>0.125–64</td>
<td>-</td>
<td>c</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Itraconazole (mg/L)</td>
<td>Sigma-Aldrich, UK</td>
<td>0.015–8</td>
<td>-</td>
<td>c</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Calcium hydroxide</td>
<td>Sigma-Aldrich, UK</td>
<td>12.5</td>
<td>0.55</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chlorhexidine digluconate</td>
<td>GlaxoSmithKline, GE</td>
<td>6.0</td>
<td>1.8</td>
<td>a,b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorhexidine digluconate</td>
<td>Curaprox, UK</td>
<td>6.0</td>
<td>0.9</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>1.8</td>
<td>-</td>
<td>a,b,c,d</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chlorhexidine acetate</td>
<td>Univ. Pharmacy, FI</td>
<td>6.0</td>
<td>0.17</td>
<td>-</td>
<td>-</td>
<td>a</td>
<td>-</td>
</tr>
<tr>
<td>Chlorhexidine digluconate</td>
<td>Univ. Pharmacy, FI</td>
<td>6.0</td>
<td>0.17</td>
<td>-</td>
<td>a</td>
<td>a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thiglycollate</td>
<td>Oxoid, UK</td>
<td>5.2</td>
<td>a</td>
<td>a,b</td>
<td>b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Sigma-Aldrich, UK</td>
<td>7.0</td>
<td>c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Horse serum</td>
<td>Oxoid, UK</td>
<td>5.2</td>
<td>-</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peptone water</td>
<td>Difco, USA</td>
<td>7.0</td>
<td>1.7</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sterile saline</td>
<td></td>
<td>7.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Brain heart infusion</td>
<td>BD, FR</td>
<td>7.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Brucella agar</td>
<td>BD, UK</td>
<td>6.7</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fastidious anaerobe agar</td>
<td>LAB-M, UK</td>
<td>7.2</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tryptic soy agar</td>
<td>Difco, USA</td>
<td>7.3</td>
<td>-</td>
<td>-</td>
<td>a</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Blood agar</td>
<td>Oxoid, UK</td>
<td>7.4</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysed blood agar</td>
<td>BD, UK</td>
<td>7.0</td>
<td>-</td>
<td>b</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sabouraud agar</td>
<td>Oxoid, UK</td>
<td>5.6</td>
<td>-</td>
<td>a,b,c,d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dentine powder</td>
<td>Univ. Oslo, NO</td>
<td>1–100</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.87–187</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>Bio-Rad, DE</td>
<td>1–100</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma-Aldrich, UK</td>
<td>1–100</td>
<td>-</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187</td>
<td>-</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Autoclaved human dentine powder (DP) prepared according to the previously published model (Haapasalo et al. 2000, Portenier et al. 2001) was kindly provided by Dr. I Portenier (University of Oslo). Briefly, extracted human third molars were kept in 5 mg/mL NaOCl to remove soft tissue and prevent bacterial growth, rinsed and autoclaved (121°C, 15 min) in distilled water to remove sodium hypochlorite. The crowns were removed with a diamond saw (Accutom, Struers, Denmark). The roots were crushed with a mixer mill (Retsch, Haan, Germany) to obtain powder with 0.2–20 µm particle size. DP was stored dry at room temperature to avoid contamination until use. Hydroxyapatite (HA) and bovine serum albumin (BSA) were tested as comparators for DP.

4.2 Methods

4.2.1 Susceptibility testing of bacteria and fungi against HICA

The standard methods, modifications, density of microbial inoculums, incubation volume, and breakpoints for detection of the results are listed in Table 5. Broth medium, pH, DL-HICA concentrations, and microbial inoculum were selected based on pilot studies. Cell densities of bacteria and yeasts in suspensions were estimated with McFarland standard and confirmed by quantitative plate counts. The density of Aspergillus spores was confirmed by cell cytometry. Solutions of 20, 40, and 80 mg/mL of HICA were prepared in broth, all at pH 5.2. Controls free of antimicrobial agents were included in all experiments. 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 cultures in microtiter plates were scanned at 0, 18, 24, 36, and 48 h, and the cultures in anaerobiosis at 0, 48, and 72 h. The inhibitory concentration of HICA that prevented 50% and 90% of the bacterial or candidal growth (IC₅₀ and IC₉₀) was determined by changes in optical density. The growth of Aspergillus isolates was determined by 100% inhibitory concentration (IC₁₀₀) of the growth by visual inspection. The tests were done with three to five replicate wells and repeated: 2-ketoisocaproic acid (KICA) and leucine were used once as comparators. The results of spectrophotometrical analyses are presented as means and standard error of the mean (SEM) of two experiments.
The microbicidal activity of HICA and comparators was observed by culture on agar after final scanning for *Candida* species and after visual inspection for *Aspergillus* species. Twenty µL of test suspension from representative wells were cultured on agar for 48 h and four days for anaerobic bacteria. Microbial growth was recorded as present or absent. The results of the microbicidal activity are shown as minimum bactericidal and fungicidal concentration (MBC/MFC). The breakpoints for detection are listed in Table 5. In the susceptibility tests, the microbicidal value was set to >99.9% killing. In the suitability tests, bactericidal value was set to 100% killing.

Susceptibility of *Candida* isolates was tested to fluconazole and *Aspergillus* isolates to itraconazole (Study IIc). The growth of *Candida* species was determined spectrophotometrically (OD 490) and *Aspergillus* species was detected by visual inspection. The minimum inhibitory concentration (MIC) was the lowest fluconazole concentration, which reduced the optical density by 50% (IC 50) in comparison to the drug-free control at 48 h. The MIC for itraconazole was the concentration, which prevented the growth of *Aspergillus* species visually (IC 100) at 48 h. The results of the impact of azole on fungal growth are presented as ICs.

In the germination study (Study IId), the impact of increasing concentrations of HICA on hyphal formation of *C. albicans* and *A. fumigatus* in horse serum and on their cellular morphology was visualised by Blankophor staining (100 nM; Blankophor, Leverkusen, Germany) in a similar manner. In a pilot study, the impact of KICA on *C. albicans* hyphal formation was tested in the same manner and only Gram staining was done. Test-solution samples (5 µL) were used. The morphology of the fungal cells was observed under bright field and fluorescence microscopy (490 nm excitation and 510 nm emission Nikon Eclipse 80i, Nikon, Japan).
Table 5. Summary of the reference methods, the densities of the microbial inoculums, incubation volumes, and breakpoints for detection of inhibitory concentrations (IC), minimum bactericidal or fungicidal concentrations (MBC/MFC).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Organism</th>
<th>Method</th>
<th>Inoculum (cfu/mL; range)</th>
<th>Volume (μL)</th>
<th>Breakpoints (IC/MBC or MFC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-HICA</td>
<td>Aerobic bacteria (Ia)</td>
<td>ASM manual(^1)</td>
<td>10⁴ (2.5x10^3–5.9x10^4)</td>
<td>200</td>
<td>24h / 48h</td>
</tr>
<tr>
<td></td>
<td>Anaerobic bacteria (Ib)</td>
<td>CLSI M11–A7(^1)</td>
<td>10⁵ (2.0x10^5–1.0x10^7)</td>
<td>200</td>
<td>48h / 72h</td>
</tr>
<tr>
<td></td>
<td>Candida (Iia)</td>
<td>CLSI M27–A3(^1)</td>
<td>10⁵ (1.1x10^5–1.1x10^6)</td>
<td>200</td>
<td>24h / 48h</td>
</tr>
<tr>
<td></td>
<td>Aspergillus (Iib)</td>
<td>EUCAST(^1)</td>
<td>3x10⁴ (cell cytometry)</td>
<td>200</td>
<td>48h / 48h</td>
</tr>
<tr>
<td></td>
<td>C. albicans (IId)</td>
<td>Germ tube testing</td>
<td>10⁶ (4.8x10^6–2.0x10^7)</td>
<td>200</td>
<td>24h / 48h</td>
</tr>
<tr>
<td></td>
<td>A. fumigatus (IId)</td>
<td>Germ tube testing</td>
<td>10⁴ (cell cytometry)</td>
<td>200</td>
<td>48h / 48h</td>
</tr>
<tr>
<td>FLU</td>
<td>Candida (Ilc)</td>
<td>CLSI M27–A3</td>
<td>10⁵ (1.1x10^5–1.1x10^6)</td>
<td>200</td>
<td>24h / 48h</td>
</tr>
<tr>
<td>ITR</td>
<td>Aspergillus (Ilc)</td>
<td>EUCAST</td>
<td>3x10⁴ (cell cytometry)</td>
<td>200</td>
<td>48h / 48h</td>
</tr>
</tbody>
</table>


4.2.2 Suitability testing of HICA for root canal medication

The reference methods, microbial inoculums, incubation volumes, and breakpoints for the detection of bactericidal effect, which were used in the suitability testing of HICA for root canal medication, are listed in Table 6. Some modifications were performed in comparison to reference methods. In Study IIIa, 48 h detection was added to the dentine powder method. In Study IIIb, potential inhibitors were added to the antimicrobial solutions (Study Ia). In Study IV microbial growth was...
detected in the root blocks departing the reference method of experimental root canal infection model.

Table 6. Summary of the reference methods, the final densities of the microbial inoculums, test volumes, and breakpoints used in the suitability testings of HICA.

<table>
<thead>
<tr>
<th>Nutritional conditions</th>
<th>Organism</th>
<th>Method</th>
<th>Inoculum (cfu/mL; range)</th>
<th>Volume (μL)</th>
<th>Breakpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient (IIIa)</td>
<td><em>E. faecalis</em></td>
<td>Dentine powder model*</td>
<td>3x10⁸ (2.8–6.3x10⁹)</td>
<td>150</td>
<td>48h</td>
</tr>
<tr>
<td>Rich (IIIb)</td>
<td></td>
<td>ASM Manual</td>
<td>1x10⁶ (1.0–2.5x10⁶)</td>
<td>200</td>
<td>48h</td>
</tr>
<tr>
<td>Deficient (IV)</td>
<td></td>
<td>Root canal infection model*</td>
<td>1.5x10⁸ (not cultured)</td>
<td>3.8</td>
<td>7 days</td>
</tr>
</tbody>
</table>


Antibacterial activity in the presence of potential inhibitors (IIIa, IIIb)

In the nutrient-deficient conditions (Study IIIa), the impact of three potential inhibitors present in human dental root canals on the antibacterial activity of HICA solution: DP suspension, HA suspension, and BSA solution was tested. The antibacterial activity of DP without antimicrobials was observed also for control.

All antimicrobials and inhibitors were prepared in sterile deionised water. Each antimicrobial agent (50 μL) was pre-incubated with each potential inhibitor (50 μL) and controls in sealed Eppendorf tubes (2 mL) for 1 h. *E. faecalis* suspension (50 μL) was added to antimicrobial-inhibitor solutions and suspensions. They were vortexed thoroughly and incubated for 48 h. All antimicrobials and controls could be mixed well with DP and HA forming homogenous suspensions. BSA was mainly in solution, but with Ca(OH)₂ slurry it formed an insoluble precipitation, which could not be analysed.

The samples were taken by a standard loop (10 μL) into peptone water (990 μL) after careful mixing at 0, 1, 24, and 48 h. They were further diluted in ten-fold serial dilution to 10⁻⁴ (detection limit) for viability testing. 20 μL of each dilution was cultured on TSA and incubated for 24 h. Colonies were counted and the purity of the cultures was checked under stereomicroscope. The results were expressed as
relative viable counts (cfu%) with standard deviations. The tests were performed once in three analogue experiments.

In the nutrient-rich conditions (Study IIIb), the influence of potential inhibitors on increasing concentrations of HICA was tested by a modification of the broth microdilution method for bacterial susceptibility testing used in Study I. The potential inhibitors were added and one dilution higher concentrations of HICA were used to test a hundred times higher density of microbial suspensions (Table 6). Two strains of *E. faecalis* were used in this experiment. The final test concentrations of antimicrobials and potential inhibitors are summarised earlier in Table 4. The positive control was commercial broth-soluble CHG 2 mg/mL diluted 1:1 in the broth. The amounts of 1.11 mg, 11.1 mg and 111.1 mg of potential inhibitors were added into 1 mL of antimicrobial-broth solutions. Minimum bactericidal concentration (MBC) for HICA was determined by culture (20 µL) on blood agar at 1, 24, and 48 h. The purity of the cultures was checked. The tests were performed once with three replicate wells.

*Antibacterial activity in human dental root canals (IV)*

Altogether 310 human vital teeth were collected at the Social and Health Services of the City of Lahti during 2010–2012, after receiving patient’s informed consent. The majority of the teeth were third molars and extracted mainly for malposition reasons. Only teeth with a clinical diagnosis of vital pulp tissue were accepted and the teeth with infected roots were excluded. This was rechecked thoroughly during the preparation of root blocks. The experimental infection model is summarised in Fig. 2. After extraction, teeth were cleaned with tap water and stored frozen until used. Root cementum was removed with a Carborundum drill, and one of the roots of multi-rooted teeth was separated with a diamond drill. Standard root blocks with root canals in 0.9 mm diameter were prepared with Gates-Glidden (GG) drill #3. All drilling was done under water-cooling. Altogether 73 root blocks with no apparent isthmus or lateral canals were accepted for the final testing. After smear-layer removal and autoclaving of the root blocks they were stabilised in six-well microtiter plates (Costar, Corning Incorporated, Corning, NY, USA) with dental wax (Astynax, Associated Dental Products, Wiltshire, UK) in random order. Root canals were dried with paper points (Orbis, Pontault Combault, France) before filling them with *E. faecalis* suspension in BHI.

During 21 days of infection, a fresh bacterial suspension was delivered every second day and BHI broth in the days between. The roots were incubated in 5%
CO₂ at 37°C. Five roots were randomly chosen for histology. After 48 h preservation in 4% paraformaldehyde, the root blocks were dehydrated in ethanol-xylene dilution series and demineralised for 90 days in 17% EDTA. The root blocks were rehydrated in xylene-ethanol dilution series and were stabilised into paraffin. Then they were cut up into 3 μm sections with a mechanical microtome and stained by the Brown and Brenn method (Brown & Brenn 1931, American Registry of Pathology 1968). The remaining root blocks were randomised in four test groups (n=17/group). Root canals were dried with paper points after irrigation with 10 mL of sterile saline. Antimicrobials were prepared in deionised water. CHG was used as a positive control. Sterile saline was an inactive negative control. Test reagents were delivered once into root canals, which were closed tightly with Relyx Unicem (3M Espe, Dental Products, Seefeld, Germany). They were incubated further in the same conditions for seven days.

Sterile instruments and trays were used in all phases of the method for performing strict asepsis, and ocular loops were used. Roots were not disinfected with any additional chemicals. The fillings were removed before sampling. Root canals were irrigated with 10 mL of sterile saline and they were dried with paper points. After detachment from dental wax, the root blocks were attached tightly with sterile locking instruments for each sampling. The inner and deeper root canal dentine samples (first 0.1 mm, second 0.1 mm) were collected separately with GG#4 (diameter 1.1 mm) and GG#5 (diameter 1.3 mm) drills into 2 mL of BHI after irrigation and drying of the canals. Each dentine sample was collected using a separate sterile drill from root canal walls by pushing the drill gently down and up and using low frequency (<1000 r/min). The surface of the drill was checked visibly to be clear after the dentine waist was delivered and mixed into BHI (2 mL) in Eppendorf tubes. Dentine in broth was vortexed carefully. The residual roots were also cultured in BHI. After 24 h incubation, 200 μL of each sample was delivered to 96-well microtiter plates for the spectrophotometric analyses (OD490). Bacterial growth was observed as changes in turbidity. In addition, 20 μL of all test solutions were cultured on lysed blood agar for viability testing and checking of the culture purity. Bactericidal activity was evaluated in the inner and deeper dentine and in the residual roots. The results of spectrophotometric analyses were depicted as bacterial growth inhibition (%) in comparison to negative control. Bactericidal activity was reported as no growth percentage of samples in the broth and in the culture on the agar.

The data did not follow a normal distribution (Study IV). Therefore, non-parametric methods were used for statistical analysis. The analysis of the OD values
between the groups of different sampling depths was done with Kruskal-Wallis test, and with Friedman and Wilcoxon Signed-rank tests between the different sampling depths within each group. The analysis of bacterial growth between the groups in different sampling depths was performed with Pearson Chi-Square and Mann-Whitney test. Between the different sampling depths the analyses were performed with Cochran and McNemar tests within each group.

Fig. 2. Main stages of the antibacterial activity testing of 400 mg/mL of DL-HICA, 400 mg/mL of Ca(OH)$_2$, 20 mg/mL CHG and 9 mg/mL of sterile saline in experimentally-infected human dental root canals ex vivo. (IV, reprinted with permission John Wiley & Sons.)
5 Results

5.1 Susceptibility of microbial isolates to HICA

5.1.1 Antibacterial activity (I)

HICA inhibited the growth of facultatively aerobic bacteria in a dose-dependent fashion, which was not observed in anaerobically cultured isolates (Fig. 3 and 4). In addition, it showed a time-dependent effect on bacterial growth of most isolates. The antibacterial activity of HICA against the gram-positive bacteria in subcidual concentrations was reduced with time. For \textit{P. aeruginosa}, the antibacterial activity increased with time at all concentrations. In the incubations of \textit{E. coli} or the anaerobically cultured bacteria, no time-dependent effect by HICA was seen. IC$_{50}$ and IC$_{90}$ comparisons are shown in Fig. 3 and 4. The isolates of \textit{S. aureus} were most susceptible to HICA (IC$_{90}$ ≤1.1 mg/mL) at 24 h, including MRSA. IC$_{90}$ was seen at four-fold lower concentrations compared to the other gram-positive bacteria (1.1 mg/mL vs. 4.5 mg/mL). The ESBL positive \textit{E. coli} isolate was also sensitive for HICA (IC$_{90}$ ≤4.5 mg/mL). HICA was less active against the other \textit{E. coli} isolates (IC$_{90}$ ≤18 mg/mL for both). Over 90% of the growth of the reference isolate and 79% of the clinical isolate of \textit{P. aeruginosa} was reduced by 4.5 mg/mL of HICA. IC$_{90}$ was achieved for the clinical isolate at 36 h. HICA inhibited in 9 mg/mL concentration 90% of the growth of \textit{E. faecalis} and \textit{Lactobacillus} isolates.

MBC comparisons at 48 h are shown in Table 7. \textit{P. aeruginosa} and \textit{F. nucleatum} isolates were most susceptible for HICA (MBC ≤4.5 mg/mL). MBC for all \textit{E. coli} isolates was 18 mg/mL. Both of \textit{E. faecalis} and \textit{Lactobacillus} isolates were susceptible at the highest concentrations to HICA (MBC ≤36 mg/mL), in which HICA had an equal bactericidal effect as the positive control, CHG. Leucine did not have any influence on aerobically cultured bacterial isolates. In contrast, the reference strain of \textit{Actinomyces israelii} and the clinical isolate of \textit{F. nucleatum} were killed in 36 mg/mL of leucine.
Fig. 3. In aerobic conditions, the growth inhibition of a representative panel of clinical isolates of bacteria in the presence of HICA and chlorhexidine (CHX; control), including antibiotic-resistant strains of MRSA and ESBL. IC50 and IC90 of HICA for bacterial strains are shown at 18, 24, 36 and 48 h. Data is presented as means (±SEM). The reference strains and the other clinical strains showed essentially the same with two exceptions: IC90 of HICA for two other isolates of *E. coli* was higher (18 mg/mL); and IC90 of HICA for the reference strain of *P. aeruginosa* in all test concentrations was achieved six hour before (data not shown).
Fig. 4. In anaerobiosis, growth inhibition of two clinical isolates of bacteria in the presence of HICA and chlorhexidine (CHX; control). IC50 and IC90 of HICA for bacterial strains is shown at 48 and 72 h. Data is presented as means (±SEM). The reference strains showed essentially the same (data not shown).

Table 7. Summary of minimum bactericidal concentrations (MBC) for HICA and leucine (LEU) at 48 h.

<table>
<thead>
<tr>
<th></th>
<th>MBC (HICA)</th>
<th>MBC (LEU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥4.5 mg/mL</td>
<td>≥9 mg/mL</td>
</tr>
<tr>
<td></td>
<td>≥9 mg/mL</td>
<td>≥18 mg/mL</td>
</tr>
<tr>
<td></td>
<td>≥18 mg/mL</td>
<td>≥36 mg/mL</td>
</tr>
<tr>
<td></td>
<td>≥36 mg/mL</td>
<td>≥36 mg/mL</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>S. aureus</td>
<td>Streptococci</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>(incl. MRSA)</td>
<td>L. rhamnosus</td>
</tr>
<tr>
<td></td>
<td>A. israelii</td>
<td>Lactobacillus spp.</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>A. israelii</td>
</tr>
<tr>
<td></td>
<td>(incl. ESBL)</td>
<td>E. faecalis</td>
</tr>
</tbody>
</table>

5.1.2 Antifungal activity (II)

Dose-dependent inhibition induced by HICA was observed in the growth of all candidal strains (Fig. 5). C. albicans and C. dubliniensis were more susceptible to HICA than non-C. albicans Candida yeasts (C. glabrata, C. krusei and C. tropicalis). In the subcidal concentrations, antifungal activity of HICA decreased with time for most isolates. However, the activity increased for clinical isolate of C. tropicalis up to 48 h.

In IC90 comparisons, C. albicans, C. dubliniensis, and C. tropicalis were most susceptible to HICA (IC90 ≤18 mg/mL) at 24 h (Table 8). One dilution higher concentration of HICA inhibited (IC90 ≤36 mg/mL) the growth of C. glabrata and C. krusei. All isolates of Candida species were sensitive for fluconazole. A. fumigatus and A. terreus isolates were the most susceptible species of the moulds tested for HICA (Table 9). The IC100 was 36 mg/mL for all (n=6) and 18 mg/mL for
five of them. HICA was not active against two isolates of *A. flavus*. All *A. flavus* isolates were sensitive for itraconazole. Three of four isolates of *A. fumigatus* were resistant to itraconazole, but one isolate (MRCM 32221) was sensitive even in 1 mg/mL concentration.

![Fig. 5. Antifungal activity of HICA and chlorhexidine (CHX; control) for a representative panel of clinical Candida isolates. IC₅₀ and IC₉₀ are shown at 18, 24, 36, and 48 h. Data is presented as means (±SEM). The influence of HICA was essentially the same for the reference strains with one exception: *C. tropicalis* inhibition was not reduced in subcidal concentrations (data not shown).](image-url)
In MFC comparisons, 72 mg/mL of HICA was fungicidal for all *Candida* isolates at 48 h (Table 8). The reference strain of *C. dubliniensis* was killed in 36 mg/L of HICA. Fungicidal activity was similar with HICA or in 1.8 mg/mL CHG. KICA and leucine were not active against *Candida* species. The MFC for the *A. fumigatus* and *A. terreus* isolates was ≤36 mg/mL of HICA (Table 9), but *A. flavus* isolates were not killed. Leucine was inactive for *Aspergillus* species up to 18 mg/mL, which was the highest test concentration.

**Table 8.** Antifungal activity of fluconazole (FLU), 2-hydroxyisocaproic acid (HICA), 2-ketoisocaproic acid (KICA) and leucine (LEU) are shown. Minimum inhibitory concentration (MIC/24 h) for fluconazole was detected by spectrophotometric analyses and minimum fungicidal concentration (MFC/48 h) for HICA, KICA, and LEU on agar.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Observation</th>
<th><em>C. albicans</em></th>
<th><em>C. dubliniensis</em></th>
<th><em>C. tropicalis</em></th>
<th><em>C. glabrata</em></th>
<th><em>C. krusei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU</td>
<td>MIC (mg/L)</td>
<td>≤0.5</td>
<td>≤0.25</td>
<td>≤1</td>
<td>≤16</td>
<td>≤32</td>
</tr>
<tr>
<td>HICA</td>
<td>MFC (mg/mL)</td>
<td>72</td>
<td>≤72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>KICA</td>
<td>MFC (mg/mL)</td>
<td>&gt;72</td>
<td>&gt;72</td>
<td>ND</td>
<td>&gt;72</td>
<td>&gt;72</td>
</tr>
<tr>
<td>LEU</td>
<td>MFC (mg/mL)</td>
<td>&gt;72</td>
<td>&gt;72</td>
<td>&gt;72</td>
<td>&gt;72</td>
<td>&gt;72</td>
</tr>
</tbody>
</table>

ND: not determined

**Table 9.** Antifungal activity of itraconazole (ITR), 2-hydroxyisocaproic acid (HICA), and leucine (LEU) are shown. At 48 h, fungal growth reduction of 100% (IC100) was determined by visual inspection of the culture broth. Minimum fungicidal concentration (MFC) was detected by culture on agar.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Observation</th>
<th><em>A. fumigatus</em></th>
<th><em>A. terreus</em></th>
<th><em>A. flavus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>ITR</td>
<td>IC100 (mg/L)</td>
<td>&gt;8</td>
<td>nt</td>
<td>0.5</td>
</tr>
<tr>
<td>HICA</td>
<td>IC100 (mg/mL)</td>
<td>≤36</td>
<td>18</td>
<td>&gt;72</td>
</tr>
<tr>
<td>MFC</td>
<td>(mg/mL)</td>
<td>≤36</td>
<td>≤36</td>
<td>&gt;72</td>
</tr>
<tr>
<td>LEU</td>
<td>MFC (mg/mL)</td>
<td>&gt;18(^1)</td>
<td>&gt;18(^1)</td>
<td>&gt;18(^1)</td>
</tr>
</tbody>
</table>

\(^1\)The highest test concentration

*C. albicans* germ tube formation was inhibited in 18 mg/mL of HICA and was prevented in 36 mg/mL as observed in Gram staining (Fig. 6). In a pilot study, the effect of 72 mg/mL of KICA was similar to 18 mg/mL of HICA. The morphology of *C. albicans* cells incubated in 72 mg/mL of HICA or in the positive control CHG was similar. The intensity of Blankophor cell wall staining was also decreased. The size of the *Candida* daughter cells was diminished at 36 mg/mL of HICA. The filamentation and sporulation of *A. fumigatus* was inhibited in 36 mg/mL and prevented at 72 mg/mL.
Fig. 6. The impact of HICA on *Candida albicans* (D17) hyphal formation and expression of the cells by Gram staining in the negative control of horse serum (a), 72 mg/mL of KICA (b), 36 mg/mL of HiCA (c), and 72 mg/mL of HICA (d), and in 1.8 mg/mL of CHG, at 24 h (a–c) and at 48 h (d, e). The influence of 72 mg/mL of KICA on germ tube formation was similar to 18 mg/mL of HICA.

5.2 Suitability of HICA for root canal medication

5.2.1 *Antibacterial activity with potential inhibitors (III)*

*Growth conditions and antibacterial activity without inhibitors*

Nutrient-deficient conditions had an impact on bacterial growth in Study IIIa. Bacterial counts decreased to hundred-fold ($10^7$ cfu/mL) immediately after mixing at 0 h, which was verified by culture control on agar. Thereafter, in the negative control of peptone water, bacterial viability reduced to 28% at 24 h and to 23% at
48 h from the initial counts, which demonstrated the nutrient-deficient test conditions (Fig. 7). In comparison to maximum bacterial growth in the negative control, HICA showed limited antibacterial effect with 73% of reduction at 1 h, but it completely prevented bacterial growth as well as chlorhexidine acetate (CHA) or CHG at 24 and 48 h. In contrast, Ca(OH)₂ slurry showed good antibacterial activity (93.5–98.5%) at 1 and 24 h, but the activity was totally lost in 48 h incubation (Fig. 7). Ca(OH)₂ solution showed moderate reduction of bacterial viability (74%) at 1 h, but it was inactive after that.

**Fig. 7. Antibacterial activity of antimicrobials at 1, 24, and 48 h.** Means of relative viable counts are demonstrated with standard deviations. Control: 1.7 mg/mL of peptone water, Ca(OH)₂ so: 0.55 mg/mL of calcium hydroxide solution, Ca(OH)₂ sl: 100 mg/mL of calcium hydroxide slurry, HICA: 33.3 mg/mL of 2-hydroxyisocaproic acid, CHA: 0.17 mg/mL of chlorhexidine acetate, CHG: 0.17 mg/mL of chlorhexidine digluconate. (10 mg/mL = 1%)

**Impact of dentine on antibacterial activity**

Human DP reduced antimicrobial activity of HICA in a time- and dose-dependent manner. The highest concentration of DP (187 mg/mL) prevented the bactericidal effect of HICA against *E. faecalis*. In contrast, 1.87–18.7 mg/mL of DP inhibited 0–3% of its antibacterial activity in comparison to the maximum bacterial growth.
in peptone water control at 48 h. (Fig. 8a). Ca(OH)$_2$ slurry demonstrated better antibacterial activity with DP than without it, especially in 18.7–187 mg/mL concentrations. The positive effect was lost with the lowest DP concentration at 48 h. (Fig. 8b). The activity of Ca(OH)$_2$ solution was 91.5% with 187 mg/mL DP at 48 h, but with lower concentrations of DP it was ineffective (data not shown). DP did not notably inhibit CHA (<1%, data not shown) or CHG (0%) (Fig. 8c). Interestingly, the highest concentration (187 mg/mL) of DP without antimicrobials decreased bacterial growth ≥86% in comparison to viable bacterial cells in peptone water control after 24 h incubation (Fig. 8d).

![Graphs showing antibacterial activity of different treatments over time.](image_url)

**Fig. 8.** Impact of dentine powder (DP 0.0–187 mg/mL) on antibacterial activity of HICA solution (a), Ca(OH)$_2$ slurry (b), CHG solution (c) and with no antimicrobials (d) against *E. faecalis* (T-75359) at 1, 24 and 48 h. The negative control was 1.7 mg/mL of peptone water. Mean relative viable counts are presented with standard deviations (SD). Detection limit: 0.01%.
Impact of hydroxyapatite or bovine serum albumin on antibacterial activity

At the end of incubation, HICA reduced >95% of bacterial growth in comparison to negative control in spite of the presence of 187 mg/mL of HA (Fig. 9a). An error in the diagram of the original article describing the interactions between HICA and HA is corrected in Fig. 9a. In the presence of BSA, the activity of HICA was eliminated and the activity of CHG was inhibited less than 11% after 24 h (Fig. 9b). CHG showed ≥99% reduction of bacterial viability with HA at all time points. The activity of CHA was inhibited by HA and BSA at first, but after 24 h incubation the activity was normal. Ca(OH)₂ solution and slurry were inactive with both of these reagents. (Fig. 9a, 9b), and slurry precipitated with BSA.

Fig. 9. In nutrient-deficient conditions, the impact of 187 mg/mL hydroxyapatite (a) or bovine serum albumin (b) on the antibacterial activity of antimicrobials is presented. Mean relative viable counts (%) and standard deviations (SD) are depicted. Control: 1.7 mg/mL of peptone water, Ca(OH)₂so: 0.55 mg/mL of calcium hydroxide solution, Ca(OH)₂sl: 100 mg/mL of calcium hydroxide slurry, HICA: 33.3 mg/mL of 2-hydroxyisocaproic acid, CHA: 0.17 mg/mL of chlorhexidine acetate, CHG: 0.17 mg/mL of chlorhexidine digluconate. Detection limit: 0.01%.

Impact of inhibitors on antibacterial activity in nutrient-rich conditions

In nutrient-rich conditions, thiglycollate broth was used as bacterial growth media in all test solutions and therefore it was the negative control of Study IIIb. HA and BSA reduced HICA’s antibacterial activity in a dose-dependent manner at 48 h (Fig. 10). The most active inhibitor was HA and the least active was BSA. MBC of HICA increased to 72 mg/mL with 1 mg/mL of HA, 10 mg/mL of DP or BSA. Antibacterial effect of HICA was prevented by 100 mg/mL of DP or 10 mg/mL of HA.
Fig. 10. In nutrient-rich conditions, minimum bactericidal concentration (MBC) of 36–108 mg/mL of HICA against *E. faecalis* with potential inhibitors (1–100 mg/mL): dentine powder (DP), hydroxyapatite (HA) or bovine serum albumin (BSA), and without them (Control) at 48 h.

5.2.2 Antibacterial activity in dental root canals ex vivo (IV)

One root block was excluded from histological analyses because of incomplete demineralisation, which affected the cutting, attachment, and staining of the sections. Invasion of *E. faecalis* into dentine tubules over the depth corresponding to the sampling depth of 0.2 mm from pulpal wall surface was observed in all roots (Fig. 11). Penetration of bacterial cells was discontinuous in the tubules. On histological analysis bacteria were seen to invade beyond 1.0 mm from the pulpal wall in all roots examined.
**Bacterial growth inhibition**

In comparison to sterile saline treatment, HICA inhibited 43% of *E. faecalis* growth in the inner dentine (first 0.1 mm), 91% in deeper dentine (second 0.1 mm), and 21% still in the residual roots after 7 days incubation (Fig. 12). Respectively, CHG inhibited 56% of the growth in the inner dentine, 43% in the deeper dentine and was fully inactive in the residual roots. Ca(OH)$_2$ did not show any antimicrobial activity in comparison to sterile saline (negative control). In addition, HICA and CHG inhibited the bacterial growth in the inner and deeper dentine significantly better than in the residual roots (p<0.01; Wilcoxon Signed Rank test).
In spectrophotometric analyses, mean bacterial growth inhibition (%) with standard error of mean (SEM) in comparison to sterile saline treatment are shown in the inner dentine (≤0.1 mm from root canal surface), deeper dentine (0.1–0.2 mm from root canal surface) and in the residual roots after medication with 400 mg/mL of HICA paste, 400 mg/mL of Ca(OH)₂ paste or 20 mg/mL of CHG solution (n=17/group). (IV, reprinted with permission from John Wiley & Sons.)

Bactericidal activity

In the inner dentine, HICA or CHG medication resulted in a bactericidal effect and roots free of bacterial growth in 82% or 88% of the samples (Fig. 13). In 59% of Ca(OH)₂ and 65% of sterile saline samples, no growth was detected. In the deeper dentine, bactericidal effect was found in 100%, 88%, 76%, and 71% of HICA, CHG, Ca(OH)₂, or sterile saline samples, respectively. The differences between the groups in the inner or deeper dentine were not statistically significant. In the residual roots, 29% of HICA and 18% of CHG samples showed bactericidal effect, whilst no sample with Ca(OH)₂ and sterile saline treatment were free of bacterial growth. The difference between HICA and Ca(OH)₂ or sterile saline groups were significant (p=0.008; Pearson’s Chi-Square test with Mann-Whitney pairwise comparison),
but not between CHG and Ca(OH)$_2$ or saline groups. (Fig. 13). The results of viability testing on agar showed essentially the same, in which 35% of residual roots with HICA medication were successfully disinfected and no growth was observed. The results of viability testings are in line with the results of spectrophotometric analyses in the present study.

![Graph showing no growth percentages](IV, reprinted with permission from John Wiley & Sons.)

**Fig. 13.** In spectrophotometric analyses, relative amount of samples with no bacterial growth after medication with 400 mg/mL of HICA paste, 400 mg/mL of Ca(OH)$_2$ paste, 20 mg/mL of CHG solution and 9 mg/mL of sterile saline (n=17/group) is shown in the inner dentine (≤0.1 mm from root canal surface), deeper dentine (0.1–0.2 mm from root canal surface), and residual roots in broth. (IV, reprinted with permission from John Wiley & Sons.)
6 Discussion

This series of studies were performed in order to gain insight regarding the antimicrobial spectrum of HICA and its potential for root canal medication. The hypothesis of the study that HICA is a potential antimicrobial agent for the treatment of topical infections was tested and found to be correct within the limitations of in vitro and ex vivo methodology. The results indicate that HICA has broad-spectrum antimicrobial activity. It is bactericidal against several species of gram-positive and gram-negative cocci or rods in aerobic and anaerobic test conditions. It is fungicidal against yeasts and some species of spore forming moulds. HICA inhibits the hyphal formation and filamentation capacity of dimorphic fungi, which are considered to be important virulence factors for these species. The interactions between HICA and dentine that affect the antibacterial effect are dose-dependent and moderate. In comparison to presently-used root canal medicaments, HICA has better long-term antibacterial activity in infected human dental root canals. The principle findings of the study are discussed with the methodological considerations, and the results are compared to the earlier literature.

6.1 Susceptibility testing studies

Repetition of the studies is important for the assessment of the susceptibility of microbial species and the activity of antimicrobial agents. Standardization and conformity of the methods assists in the repetition and comparison of the results of different studies. E. faecalis has been widely studied since it has shown to be the most prevalent organism in persisting infections, especially over the past two decades. A PubMed search with keywords of “calcium hydroxide and Enterococcus faecalis” resulted in 223 articles from the year 1983 to date, 17 of which were clinical trials. In contrast, “calcium hydroxide and Candida” resulted in 88 studies, including one clinical study. A search with keywords of “chlorhexidine and Enterococcus faecalis” found 333 papers from the year 1969 to date, 20 of them being clinical trials. Ca(OH)2 studies were endodontic studies and in chlorhexidine studies urinary infections were widely represented. In general, biocides are less frequently studied in comparison to antibiotics. Hundreds of different clinical trials on fungal species and azoles were found. The present study series is the first to assess the susceptibility of E. faecalis to HICA, which was performed in three different environments: in nutrient-rich (Study I, II and IIIb) and nutrient-deficient conditions in vitro (Study IIIa) and in human root canals ex vivo (Study IV). In
addition, interactions of HICA with potential inhibitors were observed for the first time in two different nutritional conditions *in vitro* (Study IIIa and IIIb).

### 6.2 Methodological considerations

The susceptibility studies of microbes to HICA were performed in nutritionally rich environments with broth control. Bacterial species in Study Ia and Ib, and candidal species in Study IIa were analysed according to standard susceptibility microdilution testing methods by spectrophotometer (Jorgensen *et al.* 2007, Clinical and Laboratory Standards Institute 2001, 2008). These analyses were supported by viability testing on agar. Susceptibility of spore-forming moulds in Study IIb were analysed by visual inspection (Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing 2008). Histological analyses were performed in germination study of dimorphic fungal species (Study IIb) to show the impact of HICA on hyphal formation. (Fig. 14).

The suitability studies of HICA for root canal medication were performed following a modified direct exposure method *in vitro* (Study IIIa; Haapasalo *et al.* 2000) by another modification of direct exposure method based on standard microdilution methods for comparison *in vitro* (Study IIIb; Jorgensen *et al.* 2007) and by experimental infection model *ex vivo* (Study IV; Basrani *et al.* 2002). Direct exposure methods were modified by adding potential inhibitors in Study IIIa and IIIb, in which the results were analysed by viability testing on agar. No spectrophotometric analyses could be performed in these, because of the non-soluble consistence of Ca(OH)₂ paste and potential inhibitors: DP and HA. Agar diffusion test was not accepted for these studies, because it is an unsuitable method for comparing agents with different chemical structures and with heterogeneous ability to diffuse into medium (Estrela *et al.* 2001). Histological analyses were performed in root canal infection models *ex vivo* (Study IV) to confirm the penetration of bacterial cells into dentine tubules. (Fig. 14).

Simulation of clinical conditions in the root canals is very difficult (Siqueira 2008). Despite the promising results of the present study, they do not necessarily correlate to the treatment outcomes *in vivo* and any conclusions of clinical efficacy of HICA cannot be driven so far. However, this study is the largest study of the antibacterial and antifungal spectrum of HICA and the first study about its potential for root canal medication.
Fig. 14. Experimental methods (Study I–IV, experiments a–d), exposure time of antimicrobials with microbial species, time of experimental infection (Study IV), microbial species, nutritional conditions and analyses performed are shown. ND: nutrient-deficient, NR: nutrient-rich.

6.2.1 Culture media

Culture media is an essential prerequisite for microbial growth. In general, pH, temperature, and availability of oxygen have an impact on the microbial growth, too. Thioglycollate broth was accepted for the experiments of Study I and II as a culture medium, which is suitable for both aerobic and anaerobic organisms.
Importantly, thioglycollate allowed the growth of anaerobic bacteria, which were the most demanding organisms to culture included in these studies (Clinical and Laboratory Standards Institute 2001). Mueller-Hinton broth is recommended for susceptibility testing of aerobic and facultative bacteria (Clinical and Laboratory Standards Institute 2012), and RPMI-1640 for yeasts and spore-forming moulds (Clinical and Laboratory Standards Institutes 2008, Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing 2008). Therefore, RPMI-1640 was used in susceptibility testing of fungal species to azoles (Study IIc). In Study IId, horse serum was used for induction of hyphal formation (Johnson et al. 1982). Thioglycollate broth was used also in Study IIIb to evaluate the impact of nutritional conditions on interactions of HICA and potential inhibitors. The use of same culture media in different experiments makes the results comparable to each other (Study Ia, Ib, IIa, IIb and IIIb). The results of the present studies are also comparable to other similar studies in microbiology and in development of new antimicrobial agents.

In Study IIIa, the use of peptone water for microbial suspension was adapted from the previous studies (Haapasalo et al. 2000, Portenier et al. 2001). The further dilutions (2:1) resulted in low final concentration of peptone water, which provided a nutrient-deficient test environment for all incubations in Study IIIa. Deficiency of nutrition was supported by a finding in a pilot study, in which the same concentration of peptone water reduced the E. faecalis viability by 77%, at 48 h incubation. In the root canal infection model (Study IV), three weeks incubation of root canals with bacterial suspension in brain heart infusion was also adapted from previous studies (Basrani et al. 2002, 2003). This provides bacterial penetration into dentine tubules before medication (Basrani et al. 2003, Zapata et al. 2008, Du et al. 2015). No additional culture media were used in Study IV during medication. The cultural difference between antimicrobial susceptibility testing methods in microbiology and endodontology is based on nutrient-deficient environment in root canals during root canal treatment (Sundqvist & Fidgor 2003) and nutrient-rich conditions usually in other human tissues.

6.2.2 Negative and positive controls

Negative control together with positive control forms the frame of the observation in antimicrobial activity studies. Positive control was used for microbicidal control and negative control was used for control of microbial growth in media, which allows microbes to grow freely in nutrient-rich conditions (Study I, II ad IIIb) or to
cause passive reduction in microbial viability in nutrient-deficient conditions (Study IIIa and IV). These terms are also used in an opposite manner in the literature to reflect bacterial growth or killing (Han et al. 2001, Shokraneh et al. 2014). Thioglycollate broth allowed maximum growth of the organisms in the experiments and served as a negative control in Study I, II, and IIIb. In Study IIIa, a maximum amount of residual bacteria was detected in peptone water control and in sterile saline control in Study IV, and they were used as negative controls. The main positive control of the present study series, CHG, killed all the organisms at 0.17–1.8 mg/mL concentration in a 48 h incubation in Studies I–III. In contrast, ten-fold higher concentration of 20 mg/mL of CHG failed to achieve full antibacterial effect in root canals in Study IV. This difference could be explained with the polymorphous biological environment of the intradental system.

Since saline is widely used in endodontic studies as a negative control, it is noteworthy that it fails to increase bacterial growth in long incubations in vitro (Ballantyne 1929, Valu 1965), in dental root canals ex vivo (Maekawa et al. 2013) or in vivo (Byström & Sundqvist 1983). This may be explained by normal bacterial growth curves with exponential growth, stationary state, and decline phase (Roszak & Colwell 1987). The passive effect of sterile saline on bacterial viability was also confirmed in a pilot study, in which viability of E. faecalis cells was reduced by 99% over a six-day incubation. Saline and 5% NaOCl solution have been used earlier as root canal irrigants. For toxicity reasons from high concentration of NaOCl, Byström & Sundqvist (1983) observed and showed the better antimicrobial activity of 0.5% of NaOCl solution in comparison to saline solution in root canals. In their study, after five appointments with two to four days interval and with saline medication for all subjects no bacterial growth was observed in twelve of fifteen roots (80%) with NaOCl irrigation and in eight of fifteen roots (53%) with saline irrigation. Nowadays, saline is still widely used in the treatment and prevention of wound infections (Weiss et al. 2013). In experimental infection studies of root blocks, saline is the most generally used alternative medication and negative control (Table 11; see page 89). The use of control groups without infection, without medication (Han et al. 2001, Zehnder et al. 2006), and with microbial broth could assist in the assessment of antimicrobial activities of different agents, too. In particular, the trend of reduction in bacterial viability in saline refers to long-term incubations of interappointment medication studies. This would not necessarily be seen in short-term incubations of irrigation agent studies.
6.2.3 Test organisms

For evaluating the microbial spectrum of HICA in Study I and II, representatives of seventeen different general human pathogenic organisms were chosen from facultatively aerobic and anaerobic gram-positive and gram-negative bacterial species (n=9), and from yeasts (n=5) and spore-forming moulds (n=3). At least two isolates of each microbial species were included: clinical isolate and reference strain of each bacterial and candidal species were used for control. Aspergillus species were mainly clinical isolates. Although the number of isolates from single species was restricted, the spectrum of tested organisms was a good selection of most general human pathogens. The spectrum of test organisms is a representative panel of microbial species commonly found in different phases of root canal infection and covers well the most common findings in persistent root canal infection such as *E. faecalis* and *C. albicans*. All test organisms have general importance as human pathogens. Antibiotic-resistant strains were tested because of their particular importance in the development of new antimicrobials.

*E. faecalis* was chosen as a test organism to evaluate the suitability of HICA for root canal medication in Study III and IV, because it is the most general finding in persistent root canal infections (Portenier et al. 2003, Stuart et al. 2006, Zehnder & Guggenheim 2009, Haapasalo et al. 2011, Lew et al. 2015, Zhang et al. 2015). The ability of *E. faecalis* to penetrate deep into the dentine tubules and to escape immediate contact with root canal medicament results in problems in eradication by presently-used chemo-mechanical methods. In Study I, *E. faecalis* was the most tolerant bacterial species for HICA, together with *Lactobacillus* species. Only yeasts and *A. flavus* were less susceptible to HICA.

6.2.4 Microbial cultures

Antimicrobials were exposed to planktonic organisms in Studies I, II, and III. The incubation time was mainly 48 h, but longer incubation time was only needed for anaerobic cultures to allow sufficient growth (Study Ib; 72 h). ICs and MBCs were detected following the breakpoints of standard methods. In Study IV, the medication time was one week after three weeks experimental infection. This time should have been sufficient for the bacteria to form biofilm on dentine prior to exposing them to antimicrobials as in previous studies. Bacterial biofilm formation on root canal wall has been shown to take place already at 24 h incubation (Ozdemir et al. 2010). *E. faecalis* penetration into dentine tubules and biofilm maturation has
been shown by confocal laser scanning microscopy after three weeks of experimental infection (Zapata et al. 2008, Du et al. 2015). However, this could not be detected in the study model used in the present study. In the present study the penetration of *E. faecalis* cells up to 1.0 mm from root canal surface in dentine tubules in Study IV are in line with previous reports. Bacterial invasion has been reported from 0.1 mm to 1.5 mm depth in human teeth in histological analyses, by scanning electron microscopy or by confocal laser scanning microscopy (Basrani et al. 2003, George et al. 2005, Zapata et al. 2008, Harrison et al. 2010).

The depth of bacterial invasion into dentine tubules depends on the microbial adherence, availability of nutrition and oxygen (George et al. 2005) and this may not be continuous (Love 2002, George et al. 2005). In addition, dentine tubulus wall may provide a barrier for dyes in Gram staining, which may lead to a lesser extent of visibly stained bacteria than is present in reality. Only blue-coloured round spots should be accepted for detection of coccac bacteria. Black or brownish findings may contribute to artefacts caused by air bubbles. Bacterial penetration and dentine infection was shown to be successful before medication in Study IV. Non-susceptibility of microbial cultures in dentine tubules to disinfectants could be a consequence of the lack of direct contact between antimicrobials and target organisms or due to microbial resistance in biofilms.

6.2.5 Potential inhibitors

**Hydroxyapatite**

According to previous studies, HA would be the main component of dentine, which is responsible for the inactivation of antimicrobial effect of Ca(OH)₂ in the root canal (Portenier et al. 2001, Haapasalo et al. 2007). Increasing concentrations of HA increased the MBCs of HICA in Study IIIb. In the nutrient-rich conditions (Study IIIb), the impact of HA on the antimicrobial activity of HICA was stronger than in nutrient-deficient conditions at 48 h (Study IIIa). High concentration of HA reduced the activity of HICA only by 4.3% in comparison to bacterial growth in peptone water control; but in nutrient-rich conditions, twenty-fold concentration of HA was sufficient to prevent the activity of HICA at 48 h. This may be explained by the higher amount of proteins present in thiglycollate broth (Study IIIb), in which protein binding and interactions of HA may be more probable than in low concentration of peptone water in Study IIIa. This is in line with the finding that
BSA reduced the antimicrobial activity of HICA in a dose-dependent manner in a nutrient-rich environment (Study IIIb). In nutrient-deficient conditions, the inactivation of HICA by HA was substantially less than that of Ca(OH)$_2$. In line with previous results, the antimicrobial activity of Ca(OH)$_2$ was completely eliminated by 187 mg/mL of HA in Study IIIa (Portenier et al. 2001).

**Serum albumin**

The inflammatory exudate may be the main source for albumin and other serum proteins in the infected root canals, while the levels of them are not known (Haapasalo et al. 2007). In the present study, BSA was used instead of human serum albumin or other serum proteins as in similar experiments earlier (Portenier et al. 2001, Sassone et al. 2003, Pappen et al. 2010). Therefore, the results may not precisely reflect the conditions in the root canal in clinical infection, although BSA and human serum albumin are highly uniform in structure. Earlier results were supported by a finding that Ca(OH)$_2$ solution lacked any antimicrobial effect in the presence of BSA in Study IIIa (Portenier et al. 2001). In contrast, BSA formed an insoluble precipitation with Ca(OH)$_2$ slurry and therefore they could not be analysed in Study IIIa. Although, the antimicrobial activity of HICA was prevented by 187 mg/mL of BSA in Study IIIa, 10–100 mg/mL of BSA increased the MBC of HICA only by one dilution from 36 mg/mL to 72 mg/mL in Study IIIb. This finding indicates that in spite of a possible flow of inflammatory exudate into the root canal, HICA would potentially be able to sustain its antimicrobial activity.

**Dentine powder**

Dentine remnants in root canal are probably mixed with soft tissue remnants at least at the first appointment. In addition, interactions between antimicrobials and DP may be enhanced by notably larger surface area in powder in comparison to dentine on the unbroken root canal wall. The highest test concentration of 187 mg/mL of DP would not reflect clinical conditions in the well-prepared and irrigated root canals. Earlier results were supported by a finding that Ca(OH)$_2$ solution lacked any antimicrobial effect in the presence of DP in Study IIIa (Portenier et al. 2001). In contrast, the antibacterial activity of Ca(OH)$_2$ slurry was enhanced with the highest test concentration of DP in Study IIIa. This kind of enhancement in antimicrobial activity of bioactive glass and MTA is also shown with bioactive glass and DP (Zehnder et al. 2006, Zhang et al. 2009). However, DP failed to
prevent the antibacterial activity of HICA at clinically relevant concentrations (≤18.7 mg/mL) in Study IIIa and IIIb, unlike it did in 1.87 mg/mL concentration for the activity of Ca(OH)₂ slurry in Study IIIa.

**Impact of preincubation time**

Haapasalo and colleagues (2000) also showed that increasing preincubation time (0–24 h) between antimicrobials and potential inhibitors before adding microbial suspensions to the test solution increases the interaction effect. However, this merits further research. It is noteworthy that the results of the present study similarly indicate that one hour of preincubation may enhance the interaction effects in comparison to drug-free controls. In addition, the order of test component delivery is opposite to clinical conditions, in which microbial species are present in root canals at first. Secondly, the amount of DP could be increased after preparation. Finally, antimicrobial agents are delivered into root canal during root canal treatment, especially interappointment medication is the last component used during the appointment. The interactions may happen between interappointment medication and dentine in root canal before next appointment. This order could be the same in a case of reinfection. In this context, one hour preincubation is far too short. However, preincubation of antimicrobials with potential inhibitors may aid in the assessment of differences *in vitro*.

**Comparisons and clinical relevance**

In nutrient-deficient conditions, the impact of DP on antibacterial activity of HICA was dose-dependent (Study IIIa). The effect of all potential inhibitors (DP, HA, and BSA) on the antibacterial activity of HICA was similarly dose-dependent in nutrient-rich conditions (Study IIIb). Only one concentration of HA and BSA were tested in Study IIIa as technical comparators in nutrient-deficient environment. Interactions between the tested reagents may also be bidirectional and antimicrobial activity may be reduced or enhanced. In addition, the interactions may also change the direction over the incubation time as was shown in Study IIIa between Ca(OH)₂ slurry and 1.87 mg/mL of DP.

While keeping the clinical relevance of the results in mind, the albumin concentration in human serum is 35–55 mg/mL, which is the major source of albumin in inflammatory exudate. Relative amount of HA in human dentine is 70%. Therefore, the comparisons between dentine and HA should be done in that context.
The test concentrations of \( \leq 100 \text{ mg/mL} \) of BSA and \( \leq 10 \text{ mg/mL} \) of HA in the present study may have some clinical relevance. In this context, the presence of HA would lead more probably to inhibition of HICA than the presence of serum albumin. Therefore, the results of the present \textit{in vitro} study show some basic evidence of potential interactions between present root canal medicaments or HICA and dentine, causing improved or worse antimicrobial action.

6.2.6 Human teeth

Study IV was performed in the root canals of extracted human teeth. Six mm long root blocks of the canals were prepared uniformly to minimize the natural morphological discrepancy between the roots. A total of 73 roots of initial were carefully selected from 310 teeth, excluding the roots with apparent isthmus or lateral canals. The test volume in root canals was made analogous (3.8 \( \mu \text{L} \)) in all incubations with as minimum variation as possible. No additional antimicrobials were used during the collection, preservation and preparation of the blocks. The method was practiced in pilot studies. At the commencement of experiments, sterility of the root blocks after autoclaving was confirmed by incubation in broth. During the relatively long infection and medication time, contamination of the blocks was the major concern for the success of the whole experiment. However, this was succesfully prevented with strict asepsis, which was confirmed with the purity of the culture controls on agar at the end of the study. The results of the present study can be adapted well to human root canal treatments. However, controlled clinical trials are needed to show the relevance of the results in clinical conditions.

6.2.7 Group size and statistical analysis

For appropriate statistical analyses, the amount of replicates and repeats should have been larger in Studies I–III. It is inappropriate to analyze small amounts individual isolates statistically, especially when they represent technical replicates instead of true biological replicates. The used experimental setting set-up could be improved by higher number of microbial species. Definition of MIC\(_{90}\) for a new antimicrobial agent means that the agent kills at least 90\% of different isolates (Hoogkamp-Korstanje 1997, Schwarz \textit{et al.} 2010). Therefore, the use of IC\(_{90}\) was prefered to describe over 90\% reduction of bacterial growth of single species in the present study. However, the spectrum of tested organisms in the susceptibility
testing was a representative selection of most general human pathogenic organisms (Study I and II). Studies with a large amount of single species of bacteria would be especially difficult to perform in the endodontic field, because the root canal findings consist of numberless variation of species and microbial sampling is not a routine operation during clinical root canal treatment (Molander et al. 1998, Chávez de Paz et al. 2003, 2004, Peciuliene et al. 2001, 2008, Rôças & Siqueira 2008, Siqueira & Rocs 2014).

In general, the availability of human teeth, especially of single-rooted teeth, restricts the amount and the size of the test and control groups in reality in endodontic studies. In Study IV, there were a larger amount of replicates included per group (n=17) and one repeat could be performed. Single isolate of E. faecalis was used as a technical replicate in all incubations and different human teeth represented true biological replicates in each incubation. Statistical analyses were performed as appropriate in the present study. Significant differences in antibacterial activity were seen between the group of HICA and Ca(OH)2 in residual roots in >0.2 mm depth of dentine. The present differences in the inner dentine and deeper dentine group would have been statistically significant in comparison to sterile saline treatment, if the size of test groups would have been extended by eight root blocks. However, this study series provides basic and moderate evidence of antimicrobial potential of HICA for planning of clinical investigations in the future.

6.3 Antimicrobial activity

6.3.1 HICA (I, II, III, IV)

**Susceptibility of bacteria and fungi (I, II)**

Antimicrobial HICA has broad-spectrum antibacterial and antifungal activity. Microbicidal activity of HICA arises slowly, but it is long lasting in 36 mg/mL concentration for gram-positive and gram-negative bacterial strains (Study Ia and Ib) and in 72 mg/mL concentration for Candida and Aspergillus species (Study IIa and IIb). S. aureus isolates, including MRSA were the most sensitive species for HICA with an IC90 of 1.1 mg/mL and P. aeruginosa with a MBC of 4.5 mg/mL. The most tolerant bacterial isolates belonged to Lactobacillus and Enterococcus species. The growth of Lactobacillus species and E. faecalis was reduced over 90% by 9 mg/mL of HICA. In subcidal concentrations, HICA was bacteriostatic for all
bacterial isolates, except clinical and reference strains of \( E. \text{coli} \) and \( F. \text{nucleatum} \) in Study Ia and Ib. Since MBC was 18 mg/mL for all \( E. \text{coli} \), including ESBL isolate, the present results were in line with the findings of Hietala and colleagues (1979), in which the MBC for \( E. \text{coli} \) was 4 mg/mL. It is also noteworthy that IC\(_{90}\) was 4.5 mg/mL for ESBL isolate of \( E. \text{coli} \) in the present study.

One interesting finding of a pilot study in anaerobic test conditions in pH 7.0 was that \( \leq 36 \) mg/mL of HICA was bactericidal also for both isolates of obligately anaerobic \( P. \text{gingivalis} \) detected from human gingival sulcus (T-75048 and ATCC 33277) at 48 h. MBC for \( F. \text{nucleatum} \) isolates increased to 36 mg/mL of concentration in neutral pH. For \( A. \text{israelii} \) isolates, MBC in 36 mg/mL of HICA was not achieved until 96 h incubation. \( P. \text{gingivalis} \) isolates were excluded from the present investigation, because they did not survive in the test condition of Study Ib (pH 5.8) in pure thioglycollate broth. These findings support the previous knowledge of the antimicrobial activity of HICA, which is at its best near pH 5.2 (Hietala et al. 1979). The effect is also seen in neutral pH in anaerobiosis, which was not observed in aerobic conditions for the other bacterial species.

A fungistatic effect of HICA was clearly seen for \( C. \text{albicans} \), \( C. \text{dubliniensis} \), and \( C. \text{tropicalis} \) in 18 mg/mL concentration and at least in 36 mg/mL concentration for \( C. \text{glabrata} \) and \( C. \text{krusei} \), whilst MBC was 72 mg/mL for all yeast isolates in Study IIa. IC\(_{50}\) is the detection criteria for MICs for fluconazole (Clinical and Laboratory Standards Institute 2008). When the same criterion was used, IC\(_{50}\) would be 18 mg/mL of HICA for all \( C. \text{andida} \) species. Earlier, fungicidal activity has been demonstrated in 4–10 mg/mL concentration of HICA against dermatophytic \( T. \text{cruzes} \) sp. (Hietala et al. 1979) and against spore forming moulds: \( A. \text{fumigatus} \), \( A. \text{niger} \), and \( A. \text{tubingensis} \), \( P. \text{anomala} \), \( P. \text{roquefortri} \), and \( P. \text{crustosum} \) (Broberg et al. 2007, Ndagano et al. 2011). The results of Study IIb supported previous findings of the susceptibility of \( A. \text{fumigatus} \) being \( \leq 36 \) mg/mL. In conclusion, MBCs and MFCs were marginally higher in the present study than in formerly-published studies. However, ten-fold differences in microbicidal concentrations between different studies can be explained by normal variation or minor changes in test conditions, for example in the density of original microbial inoculum.
Antimicrobial mechanisms

In Study I and II, antimicrobial activity was associated to HICA molecule and not its precursors: leucine or KICA. This is line with the findings of Ward and colleagues (1999), who showed that *E. faecalis* growth is increased in the presence of KICA, because of its ability to use KICA as an energy source. For HICA elimination, oxidation of HICA to KICA is needed. In anaerobic conditions, spontaneous oxidation reactions are naturally restricted. Gram-positive bacteria seem to react similarly with bacteriostatic inhibition in subcidal concentrations regardless of the presence or absence of oxygen. In the present study, the bacteriostatic effect of HICA was also seen under bactericidal concentrations in anaerobiosis for *A. israelii*. Strict bactericidal effect of HICA was seen against all other isolates of gram-negative bacterial species, except for the ESBL isolate of *E. coli*, for which bacteriostatic inhibition in subcidal concentrations was also seen.

The highest MBCs of HICA were seen for those species, which are known to express activity of enzymes from HicDH family. The presence of these enzymes may strengthen the tolerance of gram-positive bacteria to HICA. It may be explained by the regulation of oxidation in the presence of HicDH in *Lactobacillus* species and mandelate-DH in *E. faecalis* isolates, because of potential cross-reactions with substrates of the relative enzymes (Yamakazi & Maeda 1986, Tamura *et al.* 2002, Chambellon *et al.* 2009). Extended production of betalactamase enzymes might increase the sensitivity of gram-negative bacterial strains of *E. coli*. In contrast, *E. coli* may have potential for increased tolerance, also because of the D-HicDH coding gene in its genome (Lerch *et al.* 1989). Low oxidoreductase enzyme activity or absent microbial capacity to regulate HICA metabolism may explain the higher sensitivity of microbial species, especially in *S. aureus* (Study Ia).

Since a fungistatic effect was also seen for yeasts in subcidal concentration in Study Ia, potential impact of oxidoreductase enzymes on the antifungal effect of HICA cannot be excluded. This is supported by earlier findings of a regulator gene of HicDH in *C. albicans* (Jones *et al.* 2004, Kanehisa *et al.* 2014). In addition, HICA may have an impact on the virulence of *C. albicans* and *A. fumigatus* by inhibiting their hyphal formation (Study Iic), which is in line with the findings of Niimen and colleagues (2014a), who showed weakening of hyphal structures in terms of *C. albicans* biofilm formation in the presence of HICA.

In Study IIb, HICA was fungicidal for the majority of *Aspergillus* isolates. All *A. fumigatus* and *A. terreus* isolates were sensitive for HICA, whilst all *A. flavus*
isolates were resistant (Study IIb). Interestingly, the azole-resistant isolates were sensitive for HICA, and vice versa. This finding may contribute to both the antifungal mechanism of HICA and to the resistance mechanism of *A. flavus* for HICA. The main antifungal mode of action of azoles is inhibition of ergosterol synthesis of fungal cell membrane. Modification of this target is one possible resistance mechanism for azoles. The other ones are active efflux or changes in cytochrome P-450 enzyme (Ghannoum & Rice 1999). Therefore, these mechanisms may be involved in the tolerance of *Aspergillus* isolates for HICA, too.

Bactericidal and fungicidal activity of HICA may contribute to its amphipatic, hydrophilic, and hydrophobic properties, which allow the potential ability to reach the targets and to be active in different environments. A hydroxyl group replaces the amino group of leucine in HICA. As an amino acid derivative, HICA probably affects protein metabolism and protein structures of the cells. Connection via an amino group is needed for formation of peptide and protein chains. If HICA is attached to a protein chain instead of leucine or other amino acids, the connection of a protein chain by an amino group could be prevented. This may be a mechanism needed for protein chain reproduction in living cells, but could be destructive to a larger extent. Earlier mentioned genes and enzymes may participate in the regulation of HICA-derived reactions.

**Suitability for root canal medication (III, IV)**

The physical property of 400 mg/mL of HICA paste is suitable for the delivery and long retention in root canals. The activity of HICA against *E. faecalis* and *Candida* species is favourable to endodontic use. At least in theory, other more sensitive species may not be able to colonize root canal in the presence of active concentrations of HICA. The antibacterial effect of HICA emerges slowly, but it is long lasting. No considerable activity was seen after the first hour of incubation. It is in line with the earlier results, in which HICA had no effect against *Candida* biofilms after 12 h, but was highly active at 24 h incubation (Nieminen et al. 2014a).

A single potentially-vulnerable concentration of HICA for interactions was chosen to observe the impact of biological factors such as dentine, hydroxyapatite, and serum albumin on its antibacterial activity (Study IIIa). This concentration was equal to MBC of HICA for *E. faecalis* (Study Ia). The concentrations of comparators and controls: Ca(OH)$_2$, CHA, and CHG solutions were adapted from previous studies (Haapasalo et al. 2000, Portenier et al. 2001). In Study IIIb, dose dependence of the impact of biological factors on three increasing concentrations
of HICA was evaluated. The results indicated that increasing concentration of HICA was shown to provide more tolerance to interactions with DP, HA, and BSA. Therefore, a ten-fold higher concentration of HICA was chosen for the observations in the experimental infection study (Study IV). This dose resulted in 1.5 mg of HICA in each root canal, which is below the daily dose of threshold of concern by oral administration (1.8 mg/capita/day), according to EFSA. In Study IV, CHG was tested in a hundred-fold higher concentration and Ca(OH)₂ slurry was tested in four-fold higher concentration, which was an equal concentration to HICA paste. This concentration of CHG was comparable to that used in clinical conditions (5–20 mg/mL). Concentrations of Ca(OH)₂ slurry are generally not informed, whilst the antimicrobially active concentration of saturated solution is a temperature-dependent physical constant, being 1.45 mg/mL at 37ºC (Dean 1973). This is almost ten-fold higher than the concentration of chlorhexidine solutions in Study IIIa and over ten-fold lower than the CHG concentration in Study IV.

In the interaction study (Study IIIa and IIIb), HICA maintained its antibacterial activity in the presence of clinically relevant low concentrations of dentine (≤18.7 mg/mL) despite the change in nutritional conditions. At bactericidal concentrations, HICA was more active than Ca(OH)₂ and the activity was equal to CHA or CHG without potential inhibitors at 24–48 h. Present findings are in line with earlier results of inhibiting effect of commonly-used antimicrobials in endodontology by dentine (Haapasalo et al. 2000, 2007, Portenier et al. 2001, 2002, 2006). In addition, increasing pre-incubation time of DP together with antimicrobials increases the inhibiting effect of dentine (Haapasalo et al. 2000). Therefore, the ability of HICA to resist dentine in spite of one hour pre-incubation indicates its potential for root canal medication (Study IIIa).

In the experimental infection study (Study IV), antibacterial activities of antimicrobial agents were examined from two separate perspectives by relative comparison to negative control and by quantitative comparison of the amount of successfully disinfected roots. Relative comparisons were based on spectrophotometric analyses of broth cultures, in which antibacterial activity of HICA was higher than that of the common root canal medicaments in the deeper dentine samples (0.1–0.2 mm depth) of the root canal wall. It was similar to CHG in the inner dentine samples (≤0.1 mm depth). Quantitative comparisons were based on both spectrophotometric analyses of broth cultures and viability testing on agar. In these experiments and analyses, significantly more residual roots (depth of ≥0.2 mm) without bacterial growth was achieved with HICA treatment in comparison to Ca(OH)₂ medication, but not with CHG. HICA was the only agent, which
demonstrated significantly better antibacterial activity in comparison to sterile saline in residual roots. HICA had long-term antibacterial activity against *E. faecalis* in human dental root canals of extracted human teeth. Antibacterial activity of HICA was comparable or better than currently-used interappointment medicaments in endodontology. Regarding the results of Study III and IV, HICA could be a promising interappointment antimicrobial agent for root canal treatments.

6.3.2 Calcium hydroxide (III, IV)

In the interaction study (Study IIIa), high concentration (187 mg/mL) of HA prevented the activity of Ca(OH)\(_2\) slurry, which suggests the previous findings of HA as main responsible substance in inactivation of Ca(OH)\(_2\) by dentine (Haapasalo et al. 2007), whilst only lower concentrations of free HA in powder could reflect in clinical conditions. The antibacterial activity of Ca(OH)\(_2\) slurry was enhanced in the presence of DP in the present study, which is contrary to earlier results (Haapasalo et al. 2000, Portenier et al. 2001). The results of Study IIIa indicated that DP has moderate antimicrobial capacity by itself, which may be a good explanation for the better antimicrobial effect of Ca(OH)\(_2\) with DP. In addition, at least in clinical conditions, the water component of dentine may participate as a source of humidity in the antimicrobial action of Ca(OH)\(_2\). It is also worth noting that Ca(OH)\(_2\) slurry failed to sustain its antimicrobial activity with clinically-relevant low concentrations of free DP in Study IIIa.

In the comparison of four similarly performed studies (Table 10), all studies showed limited or no antimicrobial activity of Ca(OH)\(_2\) solution with DP in 24 h incubation with respect to the initial bacterial counts of each study (Study IV) (Haapasalo et al. 2000, Portenier et al. 2001, Athanassiadis et al. 2010). The antibacterial activity of Ca(OH)\(_2\) is based on its alkalinity, and the substitution of hydroxyl ions to the liquid phase from the slurry is needed for maintaining the high pH. Athnassiadis and colleagues (2010) showed antibacterial potential of a Ca(OH)\(_2\) slurry (Pulpdent\(^{th}\) paste) with DP against *E. faecalis* at 24 h, which is in line with present results (Study IIIa). This was also supported by a finding that the commercial Ca(OH)\(_2\) paste sustained its high pH with 187 mg/mL of DP over a one-hour incubation time, whilst the pH of Ca(OH)\(_2\) solution decreased from 12.5 to 9.0 in the same study.
Table 10. Comparison of antibacterial activity of Ca(OH)$_2$ solution (0.55 mg/mL), Ca(OH)$_2$ slurry (100 mg/mL) or commercial paste, CHA (0.17 mg/mL) and HICA (33.3 mg/mL) is shown without and with 1.87–187 mg/mL of DP at 24 h (and ≥48 h) in four representative studies in respect to initial bacterial counts of each study. In two studies only 187 mg/mL was tested. Microbial solutions were diluted into peptone water (negative control), except in one study to Tryptone soya broth. Final test concentrations of reagents (mg/mL) are listed. (10 mg/mL = 1%).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Haapasalo et al. 2000</th>
<th>Portenier et al. 2001</th>
<th>Athanassiadis et al. 2010</th>
<th>Sakko et al. 2015</th>
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</thead>
<tbody>
<tr>
<td>E. faecalis isolate</td>
<td>A197A, root canal</td>
<td>A197A, root canal</td>
<td>ATCC29212, urine</td>
<td>T-75359, root canal</td>
</tr>
<tr>
<td>Preincubation</td>
<td>0 h, 1 h, 24 h</td>
<td>1 h</td>
<td>1, 24 h</td>
<td>1, 24, 48 h</td>
</tr>
<tr>
<td>Breakpoints</td>
<td>1, 24 h</td>
<td>1, 24, 72 h</td>
<td>1, 24 h</td>
<td>1, 24, 72 h</td>
</tr>
<tr>
<td>Test agents</td>
<td>Ca(OH)$_2$ solution 0.55, CHA 0.17, (NaOCl 0.33, IPI 20/40, 2/4)</td>
<td>Ca(OH)$_2$ solution 0.55, CHA 0.17, (IPI 2/4)</td>
<td>Ca(OH)$_2$ slurry 100</td>
<td>Ca(OH)$_2$ slurry 100</td>
</tr>
<tr>
<td>Negative control</td>
<td>PW 1.7</td>
<td>PW 1.7</td>
<td>Tryptone soya broth</td>
<td>PW 1.7</td>
</tr>
<tr>
<td>Dentine powder</td>
<td>187</td>
<td>187, 187, (75, 131)</td>
<td>187</td>
<td>1.87, 18.7, 187</td>
</tr>
<tr>
<td>Activity with no DP/ DP at 24 h (≥48 h)</td>
<td>+++/−</td>
<td>+++/− (+++/-) DP 187</td>
<td>+++/− (+++/-)</td>
<td>+++/− (+++/-) DP 187</td>
</tr>
<tr>
<td>Ca(OH)$_2$ solution</td>
<td>+++/−</td>
<td>+++/− (+++/-) DP 187</td>
<td>+++/− (+++/-)</td>
<td>+++/− (+++/-) DP 187</td>
</tr>
<tr>
<td>Ca(OH)$_2$ slurry</td>
<td>ND</td>
<td>ND</td>
<td>+++/+ (+++/++) DP 187</td>
<td>+++/+ (+++/++) DP 187</td>
</tr>
<tr>
<td>HICA 33.3</td>
<td>ND</td>
<td>ND</td>
<td>+++/+ (+++/++) DP 187</td>
<td>+++/+ (+++/++) DP 187</td>
</tr>
<tr>
<td>CHA 0.17</td>
<td>+++/+</td>
<td>+++/+</td>
<td>ND</td>
<td>+++/+ (+++/++) DP 187</td>
</tr>
</tbody>
</table>

+++; very good antimicrobial activity (>99% reduction in bacterial viability in comparison to initial bacterial counts), ++: good antimicrobial activity (90–99% reduction), +: limited antimicrobial activity (50–90% reduction), -: no antimicrobial activity (<50% reduction). PW: peptone water, NaOCl: sodium hypochlorite, IPI: iodine potassium iodine, ND: not determined.

Present results of the antibacterial activity of Ca(OH)$_2$ in root canals (Study IV) are directly comparable to similar studies performed in extracted human teeth together with three weeks E. faecalis infection and seven days medication from the direction...
of root canal, which is assisted by embedding the root blocks into wax (Basrani et al. 2002, 2003) (Table 11). Otherwise, the root surface may be more predisposed for infection. The root blocks are usually cultured in the broth or without embedding them. Three weeks infection of dentine is generally accepted for a good practice to prepare experimental infection of dentine tubules providing *E. faecalis* invasion and biofilm formation (Basrani et al. 2002, 2003, George et al. 2005, Zapata et al. 2008, Du et al. 2015). Seven days medication could be an ideal timing for the second appointment, which is also generally used in clinical microbiological studies *in vivo* (Kim & Kim 2015).

In relative comparisons, no better antimicrobial activity of Ca(OH)₂ slurry against *E. faecalis* was shown in root canals compared to saline medication, which is in line with earlier results and suggestions (Basrani et al. 2002, 2003, Zehnder 2012, Lee et al. 2013, Shokraneh et al. 2014). In the comparison of thirteen studies with three weeks infection: two studies showed good antibacterial effect, five studies showed limited effect and five studies showed no effect after ≤7 days of incubation time (Table 11). The results of two studies with 14 days medication did not deviate from the line and they showed limited or no antibacterial effect of Ca(OH)₂ in culture analyses. Method of analyses may have some influence on detection of microbial cells. Delgado et al. (2010) showed smaller amount of living bacterial cells after Ca(OH)₂ medication by fluorescence microscopy in comparison to culture analyses in respect to sterile saline treatment. Kim & Kim (2014) reviewed eighteen experimental infection studies in human dental root canals with more variable periods of infection with *E. faecalis* and medication times. Twelve of them demonstrated limited activity of Ca(OH)₂ and three of them good activity, which is line with the underlying comparison (Table 11).
Table 11. Relative comparison of antimicrobial activity of Ca(OH)$_2$ against *E. faecalis* in infected human dental root canals of extracted teeth in respect to negative control (saline, water, or no medication). Studies with three weeks of infection, ≤14 days of medication and sampling methods by paper points, hand-instrumentation, or rotary-instrumentation are accepted to this comparison. The results are based on viability cultures on agar, spectrophotometric analyses of broth cultures, or viability analyses of fluorescence microscopy.

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>N/group</th>
<th>Control</th>
<th>Sampling</th>
<th>Analyses</th>
<th>&lt;7d</th>
<th>7d</th>
<th>14d</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>Han <em>et al.</em></td>
<td>12–17</td>
<td>No med.</td>
<td>PF#60,90 S</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Almyroudi <em>et al.</em></td>
<td>8</td>
<td>Saline</td>
<td>Round drills C</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>016,018,021</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Basrani <em>et al.</em></td>
<td>5–15</td>
<td>Saline</td>
<td>GG#4,5 S</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>Basrani <em>et al.</em></td>
<td>5–15</td>
<td>Saline</td>
<td>GG#4,5 S</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Krithkadatta <em>et al.</em></td>
<td>15</td>
<td>Saline</td>
<td>GG#4,5 C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Delgado <em>et al.</em></td>
<td>15</td>
<td>Saline</td>
<td>GG#5,6 C (FM)</td>
<td>- (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Kandaswamy <em>et al.</em></td>
<td>30</td>
<td>Saline</td>
<td>GG#4,5 C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Madhubala <em>et al.</em></td>
<td>8</td>
<td>Saline</td>
<td>Hi#50, PP C</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>Lee <em>et al.</em></td>
<td>12</td>
<td>Saline</td>
<td>Hi#90,110 S</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>Bhandari <em>et al.</em></td>
<td>30</td>
<td>Saline</td>
<td>GG#5 C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>Javidi <em>et al.</em></td>
<td>6–30</td>
<td>Water</td>
<td>PP, GG#5 C</td>
<td>-</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>Shokraneh <em>et al.</em></td>
<td>15</td>
<td>Water</td>
<td>GG#4,5 C</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015</td>
<td>Study IV</td>
<td>17</td>
<td>Saline</td>
<td>GG#4,5 S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**+++**: good activity with 90% reduction in bacterial viability or growth in comparison to negative control, **+**: limited activity with 50–90% reduction, -: no activity with <50% reduction, PF: ProFile, PP: paper point, Hi: hand-instrument, GG: Gates-Glidden drill, C: culture, S: spectrophotometric, FM: fluorescence microscopy.

*E. faecalis* isolates used: Han/KCCM11814, Almyroudi/59876R, Bhandari/ATCC35550, Sakko/T-75359, all other studies/ATCC29212.

In quantitative comparison of roots free of bacterial growth, Ca(OH)$_2$ prevented the bacterial growth in around 59–76% of the roots up to 0.2 mm depth, although it was not better than sterile saline treatment (Study IV; n=17). Both Ca(OH)$_2$ and saline showed limited antibacterial effect in root canals in the present study. Earlier reports based on quantitative analyses are controversial. Zehnder *et al.* (2006) showed successful disinfection in 89–100% of the roots treated with Ca(OH)$_2$ after two weeks of *E. faecalis* infection and up to 0.15 mm depth of dentine (n=9). In contrast, Basrani and colleagues (2002, 2003) showed <10% of roots free of bacterial growth to the depth of 0.2 mm (n=15). According to quantitative comparisons in the present study, the antibacterial activity of Ca(OH)$_2$ was limited.
Regarding the relative comparison to the sterile saline treatment, it was non-existent. Therefore, a better antimicrobial activity should be sought.

6.3.3 Chlorhexidine (III, IV)

DP lacked substantial influence on the antibacterial activity of CHA or CHG in 48 h incubation (Table 12), which is in line with three earlier studies performed by DP model (Portenier et al. 2002, 2006, Abbaszagedan et al. 2014). Similarly, no substantial difference between the antibacterial activity of CHA and CHG was observed in the present study. Although, delayed antibacterial effect of chlorhexidine in the presence of DP is reported earlier at 1 h incubation (Haapasalo et al. 2000, Portenier et al. 2001, 2002, 2006), which may also contribute to the preincubation time with DP beforehand. At 24 h, chlorhexidine is very active against *E. faecalis* even at a concentration of a thousand-fold that used in the studies of the underlying comparison (Abbaszagedan et al. 2014) (Table 12). In the present study, antimicrobial effect of CHA with HA or BSA, and CHG with BSA was delayed with 187 mg/mL concentration at 1 h (Study IIIa). However, such a high concentration of 187 mg/mL of free HA or serum albumin is hardly ever present in root canals, but they served as technical comparators in Study IIIa. In addition, 1 h incubation is very short in comparison to the use in interappointment medication.

In relative comparisons of Study IV, the antibacterial activity of CHG solution was limited ≤0.1 mm depth of dentine with >50% increase of reduction in bacterial growth and no effect in 0.1–0.2 mm depth with <50% increase of reduction with respect to sterile saline treatment. This is in line with the results of Lee et al. (2013), who showed that the ability of 20 mg/mL of CHG gel to eliminate bacteria is limited up to 0.2 mm depth. In contrast, Basrani and colleagues (2002, 2003) demonstrated at least good antibacterial activity of chlorhexidine solution. They also showed that chlorhexidine gel is less active than solution in human dental root canals *ex vivo.*
Table 12. Comparison of antimicrobial activity of HICA (33.3 mg/mL) and CHG solution (0.00007–0.7 mg/mL) with and without DP at 24 h (≥48 h) in representative studies is shown. In one study, the form of chlorhexidine (CHX) was not informed. The impact of 1.87–187 mg/mL of DP was examined, in three of these studies only 187 mg/mL of DP was tested. Final test concentrations of reagents (mg/mL) are shown.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Portenier et al. 2002</th>
<th>Portenier et al. 2006</th>
<th>Abbaszagedan et al. 2014</th>
<th>Sakko et al. 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>1 h</td>
<td>0 h</td>
<td>0 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Breakpoints</td>
<td>1, 24 h, 1, 24 h, 1, 4, 24 h</td>
<td>1, 24, 48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test agents</td>
<td>CHG 0.07, (IPI 1/2)</td>
<td>CHG 0.03, CHX 0.07, 0.7 (NaOCl 8, Ag NPs)</td>
<td>HICA 33.3, CHG 0.17, (Ca(OH)2 solution 0.55 Ca(OH)2 slurry 100 CHA 0.17)</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>PW 1.7</td>
<td>PW 1.7</td>
<td>PW 1.7</td>
<td>PW 1.7</td>
</tr>
<tr>
<td>Dentine powder</td>
<td>187</td>
<td>187</td>
<td>187</td>
<td>1.87, 18.7, 187</td>
</tr>
<tr>
<td>Activity with no DP/DP at 24 h (≥48 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HICA 33.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+++/- (+/++) DP187</td>
</tr>
<tr>
<td>CHG 0.00007</td>
<td>++/ND***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>+++/+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.07</td>
<td>+++/+++</td>
<td>+++/+++</td>
<td>+++/+++</td>
<td>+++/+++(+/++) DP187</td>
</tr>
<tr>
<td>0.17</td>
<td>+++/+++</td>
<td>+++/+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>+++/+++</td>
<td>+++/+++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+++: very good antimicrobial activity (>99% reduction of bacterial viability in comparison to initial bacterial counts), ++: good antimicrobial activity (90–99% reduction), +: limited antimicrobial activity (50–90% reduction), -: no antimicrobial activity (<50% reduction). PW: peptone water, Ag NPs: silver nanoparticles, NaOCl: sodium hypochlorite, ND: not determined.

In quantitative comparison of the cultures in broth and on agar, 20 mg/mL of CHG quite effectively eliminated E. faecalis from dentine up to 0.2 mm of depth, which was detected in spectrophotometric analyses and in viability testing. The finding of successful disinfection in 88% of the roots is line with earlier results (Basrani et al. 2002, 2003, Bhandari et al. 2014). In relative comparison to sterile saline, only HICA treatment could prevent all bacterial growth. This was seen in the depth of 0.1 to 0.2 mm of dentine, in the present study.
6.4 Future work

In the future, the activity of HICA could be evaluated against general human pathogenic bacterial and fungal strains by using *in vitro* biofilm models (Ceri et al. 1999, Ramage et al. 2001). HicDH enzyme expression (Broadbent et al. 2004), expression of HicDH regulating genes (Lerch et al. 1989), and enzyme activity in different microbial species could be observed (Bachmann et al. 2009). The safety of HICA for host cells such as fibroblasts and macrophages could be further evaluated in cytotoxicity assays (Mosmann 1983).

Molecular methods will broaden the knowledge of the causative organisms of root canal infections. So far, *E. faecalis* is known to be the most prevalent organism of persisting root canal infections and could be used as a primary test organism before anything else is shown. Suitability testings for endodontic use could be extended by observing the potential interactions between antimicrobials and biological factors present in root canals and their dose dependence in clinically relevant concentrations such as 1–100 mg/mL (Haapasalo et al. 2000, Portenier et al. 2001) and antimicrobial properties of dentine alone could be further evaluated (Smith et al. 2012). The viability of bacterial cells in dentine tubules could be observed in the experimental infection model of human dentine using confocal laser scanning microscopy, also after exposure to antimicrobials (Basrani et al. 2002, 2003, Zapata et al. 2008, Du et al. 2015).

For the assessment of antimicrobial activity and efficacy of HICA as a root canal medicament in clinical conditions randomized controlled clinical studies would be required (Molander et al. 2007). At first, the activity of 400 mg/mL of HICA should be compared to the most general interappointment medication, which is Ca(OH)_2. This concentration of HICA is at least ten-fold higher than the bactericidal concentration and over five-fold higher than the fungicidal concentration of HICA. In the clinical investigations, seven days is the most widely accepted period of time for medication (Kim & Kim 2015). The microbiological status of root canals is usually evaluated after the first access, immediately before the delivery of root canal medicament after chemo-mechanical preparation and after the interappointment medication. In several studies, paper point sampling (for 1 min) after gentle filing of root canal walls is used for the assessment of microbiological status of the root canals (Vianna et al. 2007, Rôças & Siqueira et al. 2011, Paiva et al. 2013, Sinha et al. 2013).

Antimicrobial activity of interappointment medication can be observed only in root canals with detected microbial infection (Sjögren et al. 1991). On the other
hand, Safavi et al. (1985) observed prevention of microbial growth during interappointment medication in root canals initially free of growth. It is worth noting that initial disinfection with ≥0.5% of NaOCl solution together with mechanical preparation may lead to ≥50% decrease of the amount of infected root canals before interappointment medication (Byström & Sundqvist 1983, Sjögren et al. 1991). Therefore, the results of chemo-mechanical preparation, interappointment medication, and combined results of chemo-mechanical preparation and interappointment medication should be reported separately. Molecular methods and culture methods could be needed for appropriate analyses of the results.

Interappointment medication is one important part of root canal treatment, since microbiological status immediately before root canal obturation predicts healing of AP (Molander et al. 2007). Therefore, the efficacy of HICA should also be studied in this context. Periapical index (PAI) scoring method could be used for following the radiological changes (Ørstavik et al. 1986). Clinical signs and symptoms should also be followed in the meanwhile. Early and late complications could probably be seen during three years of follow-up (Trope et al. 1999, Weiger et al. 2000, Waltimo et al. 2005, Molander et al. 2007). Demonstration of significant differences between interappointment medications requires large experimental groups. Subjects of one to two hundred per group are needed depending on the statistical methods used, if the effect of Ca(OH)$_2$ treatment is estimated at the level of 75% and for HICA at 90% (Study IV) and the hypothesis would be tested at the level of 0.05 with power of 0.8, for example.

6.5 Potential of HICA for other uses (I, II)

HICA has broad-spectrum bactericidal activity for several facultative and obligate anaerobic bacterial species, which indicates its potential for use in antisepsis and disinfection. Bacterial HicDH activity may reflect tolerance for HICA sensitivity. In addition to endodontic use, HICA could be a useful agent in the treatment of superficial oral and genitourinary mycosis, skin and wound infections, and disinfection of foreign body materials such as catheters, prostheses, and hearing aids (Fig. 15). In particular, the high sensitivity of staphylococcal isolates for HICA indicates its potential for the treatment of skin and foreign body infections. It could also be a useful agent in the treatment of diabetic wounds, which are often caused by E. faecalis. The activity for antibiotic-resistant strains of MRSA and ESBL may strengthen its potential for the treatment of nosocomial infections.
HICA has fungicidal activity against several *Candida* and *Aspergillus* species, which indicates its potential for the treatment of fungal infections. The influence of HICA on *C. albicans* hyphal formation and *A. fumigatus* filamentation may weaken the virulence of these human pathogens. Therefore, patients could benefit from the use of HICA in the treatment of superficial fungal oral mycositis, sinusitis, skin infections, or skin, mucosal and burn wound infections, or genitourinary infections, or in the disinfection of foreign-body materials (Fig. 15). Fungicidal activity together with bactericidal activity supports the potential of HICA for the treatment of mixed flora infections, which is typical for superficial infections in general. Importantly, the use of topical antiseptics with new mode of actions in the treatment of superficial infections instead of antibiotics may restrict the development of extensive resistance to systemic antimicrobials.

![Fig. 15. Schematic illustration of potential clinical uses of antimicrobial HICA. The root canal infection could be prevented in dental irreversible pulpitis or sterile pulpal necrosis. The infection could be treated in primary or post-treatment apical periodontitis (AP). HICA has potential also for the prevention or treatment of other superficial infections, which are listed in the four background boxes.](image-url)
7 Conclusion

Previous studies have mainly concentrated on the expression of HICA or the HicDH enzyme in single bacterial species, primarily in nutrition. The antimicrobial spectrum of HICA has not been defined previously. This study series aimed to gain some knowledge of the antimicrobial spectrum of HICA and its usefulness for the treatment of superficial infections. The interactions with potential inhibitors and antibacterial activity of HICA against *E. faecalis* in experimentally-infected dental root canals were evaluated to gain some evidence of whether HICA would be a suitable antimicrobial agent for the treatment of root canal infections as an interappointment medication. The hypotheses, that HICA could be a useful agent for the treatment of topical infections as a broad-spectrum antibacterial and antifungal agent in general and specifically it may have potential for the treatment of dental root canal infections, were accepted.

The results of the present investigation indicate that the antibacterial activity of HICA remains high despite the presence of possible clinically-relevant dentine concentrations. HICA also has long-term antibacterial activity against *E. faecalis* in human dental root canals. At bactericidal concentrations of HICA, it was more active than Ca(OH)$_2$ and in ten-fold higher concentration, the activity of HICA was at least the same as with chlorhexidine. Especially, the effect of HICA increased in the deep parts of dentine in comparison to Ca(OH)$_2$ or CHG. Saline treatment resulted in considerable decrease in bacterial growth in comparison to initial growth, which may be explained by normal bacterial growth curves and nutritional depletion. In the present studies (I–IV), HICA and CHG were clearly microbicidal for all tested organisms, except HICA for three *A. flavus* isolates. In the future, more knowledge about the activity of HICA against microbial cells in biofilms is needed (Nieminen *et al.* 2014a).

Microbial resistance to antimicrobials cause remarkable problems in the treatment of infections. The results of the present study indicate that leucine-HICA metabolic pathway could be a potential target in the development of new antimicrobials. Microbial resistance to antibiotics could be prevented by managing superficial infections with topical antimicrobials, while systemic therapy could be saved for the treatment of deep infections. Microbial tolerance to antimicrobials is a problem also in the treatment of root canal infections. New mechanisms of antimicrobials could reduce the problems in both fields.

The activity of HICA against microbial biofilms, its mode of action, enzyme expression, and enzyme activities needed for HICA-metabolism of different
microbial species remain to be clarified. For safety, cell reactions in the presence of HICA at microbicidal concentrations should be tested further before clinical studies, since an optimal root canal medication must possess good biocompatibility in case of extrusion or leakage from the root canal system to surrounding tissues. In addition, the antimicrobial potential of dentine and the compounds responsible for it should be further evaluated. Next generation sequencing techniques in microbiology may provide far more knowledge about the causative organisms of infections including root canal infections. Therefore, new strategies of investigations will arise in the future.

Regarding the results of the susceptibility studies together with anti-inflammatory properties (Nieminen et al. 2014b) of this relatively well-tolerated substance (European Food Safety Authority 2012), the present investigation provides basic evidence of HICA for general use in antiseptic and disinfection. The results of the interaction study and experimental infection study in human dental root canals indicate moderate evidence of antimicrobial potential of HICA for further investigations in endodontology.
References


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Ström K, Sjögren J, Broberg A, Schnürer J (2002) Lactobacillus plantarum Mi LAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 68: 4322–4327.


Original publications


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Original publications are not included in the electronic version of the dissertation.
1329. Lantto, Marjo (2015) Childhood mortality in Finland
1332. Petrov, Petar (2015) Leukocyte protein Trojan, as a candidate for apoptotic regulatory role
1333. Mattila, Riikka (2015) The roles of virulence factors Us3 and γ134.5 during different phases of HSV-1 life cycle
1336. Lithovius, Riitta (2015) Aspects of cleft lip and palate from Northern Finland: clefts in Northern Finland
1338. Sneck, Sami (2016) Sairaanhoitajien lääkehoitoin osaamisen ja osaamisen varmistaminen
1341. Kuoppala, Ritva (2016) Outcomes of implant-supported overdenture treatment
1342. Nanekar, Rahul (2016) Biochemical and biophysical studies on adenosine receptors and their interaction partners
Marjut Sakko

ANTIMICROBIAL ACTIVITY AND SUITABILITY OF 2-HYDROXYISOCAPROIC ACID FOR THE TREATMENT OF ROOT CANAL INFECTIONS

UNIVERSITY OF OULU GRADUATE SCHOOL; UNIVERSITY OF OULU, FACULTY OF MEDICINE; MEDICAL RESEARCH CENTER OULU; FINNISH DOCTORAL PROGRAM IN ORAL SCIENCES