DYNAMICS OF KILLING BIOFILM BACTERIA

by

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Abstract

Oral bacteria are the main cause of common oral diseases such as caries, periodontal infection and root canal infections. Bacteria in nature survive predominantly as biofilms, which are complex microbial communities composed of populations of microorganisms adhered to living or non-living surfaces and embedded in a self-produced matrix of extracellular polymeric substance (EPS). Various biofilm models have been developed to simulate the real situation in nature. In this thesis, I studied a new in vitro biofilm model, and examined the antimicrobial efficacy of current as well as newly developed endodontic irrigants/protocols against planktonic and biofilm bacteria.

Single and multispecies biofilms were grown on sterile hydroxyapatite and dentin discs coated with bovine dermal collagen Type I. Transmission electron microscopy (TEM) was used to examine the biofilm microorganisms. The antimicrobial effect of sodium hypochlorite (NaOCl), iodine potassium iodide (IPI), chlorhexidine (CHX) and a new disinfecting agent (QMiX- a mixture of CHX, EDTA, and a detergent) was evaluated. The antimicrobial strategies included in the studies were photoactivated disinfection (PAD) and its experimental modifications. Biofilms at different stages of maturation were exposed to various antibacterial agents, and the killing of biofilm bacteria was observed using viability staining and confocal laser scanning microscopy (CLSM).

The new in vitro biofilm model had similarities to in vivo biofilms, as described in the literature. This biofilm model reached maturation between two and three weeks. Mature
biofilms were less sensitive to disinfecting agents than young biofilms. The time required for the biofilms to become resistant to disinfecting agents (maturation) was not dependent on the source of biofilm bacteria or the type of disinfectant used. Modified photoactivated disinfection was up to almost twenty two times more effective in killing biofilm bacteria than conventional PAD and up to almost eight times more effective than the commonly used endodontic irrigants. A new endodontic irrigant, QMiX was more effective in killing planktonic and biofilm bacteria than 2% CHX, BioPure MTAD (a mixture of tetracycline isomer, an acid, and a detergent), and 1% and 2% NaOCl. The new biofilm model seems promising for testing and developing efficient methods to eradicate oral biofilm bacteria.
Preface

Some of the material included in this thesis has been previously published as noted below:

Papers:


This publications as well as this thesis are the principal work of the candidate, Sonja Stojicic. The thesis principal supervisor Dr. M. Haapasalo, offered editorial comments on the manuscripts and contributed advice and suggestions throughout the course of experiments that comprise these publications and this thesis. Also, Dr. M. Haapasalo is the main inventor of one of the products (QMiX) studied in the thesis. Dr. Y. Shen as co-supervisor also contributed with guidance and help in experimental design. Dr. H. Amorim was involved in the early stages of photoactivated disinfection study and performed some of the preliminary experiments (these results not included in the thesis). Dr. W. Qian and Dr. B. Johnson were involved in the development of QMiX. Dr. K. Lounatmaa provided transmission electron microscopy imaging. Focused ion beam scanning electron microscopy imaging was provided by Dr. G. Owen and Dr. Z. Wang. The candidate,
principal and co-supervisor agree that the contributions of the respective parties are as stated above.

Dr. M. Haapasalo has a conflict of interest regarding one product included in one of the studies; royalties from the sales of a root canal disinfectant QMiX.

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List of Abbreviations

AHL: acyl homoserine lactone
AI-2: autoinducer 2
AIP: autoinducing peptide
ANOVA: one way analysis of variance
AP: apical periodontitis
BHI: brain heart infusion
CFU: colony forming units
CHA: coated hydroxyapatite
CHX: chlorhexidine digluconate
CLSM: confocal laser scanning microscopy
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
EPS: extracellular polymeric substance
FIB SEM: focused ion beam scanning electron microscopy
H₂O₂: hydrogen peroxide
HA: hydroxyapatite
I₂: iodine
IPI: iodine potassium iodide
LPS: lipopolysaccharide
MB: methylene blue
MSB: mitis salivarius-bacitracin
NaOCl: sodium hypochlorite
OD: optical density
PAD: photoactivated disinfection
PCR: Polymerase Chain Reaction
PI: potassium iodide
PIT: preincubation time
PTED: post treatment endodontic disease
ROS: reactive oxygen species
rRNA: ribosomal ribonucleic acid
SEM: scanning electron microscopy
TEM: transmission electron microscopy
TSA: Tryptic Soy Agar
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Sonja Stojicic
To my twins Dušan and Vladimir
Chapter 1: Introduction
1.1 General Introduction

The bacteriological etiology of dental caries was identified over one hundred years ago by Miller (1883). Oral bacteria have also been determined to be the etiological factor in other chronic oral diseases such as periodontal (Genco 1969, Socransky 1970, Socransky & Crawford 1977, Slots 1979) and endodontic infections (Kakehashi et al. 1965, Bergenholtz 1974, Sundqvist 1976). Contrary to what occurs in acute infections, in chronic bacterial infections the planktonic phenotype generally exists only transiently and usually as a minor population with a biofilm being the causative agent. Since chronic infections are fundamentally different from acute infections, other strategies are necessary to treat the biofilm infections more efficiently. Microbial biofilms represent a protected mode of growth that allows cells to survive hostile environments and this presents a challenge for treating biofilm infections (Costerton et al. 1987, Brown et al. 1988, Anwar et al. 1992, Davies 2003, del Pozo & Patel 2007). Also, bacteria in biofilms resist host immune responses, antibiotic treatment, as well as different disinfecting agents or oral hygiene products which are efficient in treating acute infections (Brown et al. 1988, Anwar et al. 1992, Marsh 2010).

Study of the biofilm as the natural environment of bacteria has evolved tremendously in the past few decades. While bacteria were originally studied in planktonic cultures, oral biofilm models were developed to simulate the real situation in the mouth. Knowledge of biofilm characteristics is important to understand the biofilm's special behavior as well as to find ways to eliminate it (ten Cate 2006). During the last few years much emphasis has been placed on the development of novel biofilm models to facilitate the study of disinfection
and, eventually, the elimination of oral infections. Our group successfully developed a model of mixed bacterial biofilm with a presence of high numbers of spirochetes (Shen et al. 2009). This is important qualitative progress in the development of biofilm models since spirochetes are very demanding with regard to their growth requirements for in vitro culturing (Wardle et al. 1997). Spirochetes found in high numbers throughout the in vitro biofilm are one indication that this model has important similarities with in vivo biofilm.

This thesis focuses on the structure of a novel in vitro biofilm model, the effect of the source of biofilm bacteria, the level of biofilm maturation, and the type of disinfecting agents on the susceptibility of biofilm bacteria to antibacterial agents. Also, the effectiveness of eradication of biofilms and biofilm bacteria by existing and new disinfecting agents and/or protocols is evaluated. This chapter will review the etiology (Section 1.2) and microbiology of endodontic infections (Section 1.3) as well as the present knowledge of oral bacterial biofilms, including their formation, structure and the interactions between biofilm bacteria (Section 1.4). Also, endodontic treatment as a strategy for biofilm elimination from root canals will be discussed (Section 1.5). Finally, this chapter will focus on the background, hypothesis and rationale for this thesis (section 1.6).

1.2 Etiology of Endodontic Infections

All available surfaces in the oral cavity are colonized by diverse microbial biofilms. More than 700 bacterial species have been detected and considered as normal flora (Paster et al. 2001, Mager et al. 2003, Aas et al. 2003, 2005). It is estimated that about 50% of oral
species are either uncultivable or have not yet been cultivated (Paster et al. 2001). Culture independent methods are now being used to identify these species as well as to determine bacterial diversity in health and disease. The pulp and root canal system of the teeth are not exposed to the oral microflora and are usually sterile.

Oral bacteria are the main cause of endodontic infections. The bacterial etiology of these diseases was first suggested by Miller in 1894, when he demonstrated that cocci, rods and spirochetes could be found in necrotic pulps (Miller 1894). In their experiments using germ-free rats, Kakehashi et al. (1965) was the first to directly demonstrate the involvement of bacteria in endodontic infections. They showed that in germ-free rats pulpal exposure caused minimal pulpal inflammation and healing occurred despite the physical irritation and food impaction. On the other hand, pulpal exposure in the microbial environment of conventional rats resulted in severe inflammatory reaction and consequent pulp inflammation, necrosis and apical periodontitis (AP). Further confirmation of the bacterial etiology of endodontic disease came from Bergenholz (1974), Sundquist (1976) and Möller et al. (1981) who showed that apical periodontitis developed only if the necrotic pulp was infected. Möller et al. (1966) also reported that bacteria isolated from the root canals of teeth with periapical infection were able to cause apical periodontitis when inoculated in another tooth. The association of a given bacterial species with disease historically has been through the application of Koch’s Postulates. These criteria were formulated by Robert Koch in 1884 and refined and republished by Koch in 1890 (Fredericks & Relman 1996). The criteria are as follows:

1. A specific organism can be found always in association with a certain disease.
2. The organism can be isolated from the host and grown in pure culture in the laboratory.
3. The pure culture will cause the disease when inoculated into a susceptible healthy animal.
4. It is possible to recover the organism in pure culture from the experimentally infected animal.

Robert Koch’s “single species etiology” concept may not be applied for many human infections where no single pathogen but a set of species usually organized in mixed biofilm communities have been associated with disease causation. These diseases, such as caries, periodontal and endodontic diseases are polymicrobial in nature (Siqueira et al. 2012). The association of any specific species with any form of endodontic infections has not been confirmed, however, accepting the concept of “community as pathogen” it can be stated that some bacterial communities are more common in some forms of endodontic infections (Siqueira et al. 2012).

Parsek & Singh (2003) proposed four criteria to establish the causal link between biofilms and certain infectious disease. The fifth criterion was proposed by Hall-Stoodley & Stoodley (2009) and the sixth criterion was suggested by Siqueira & Ricucci (2010a). These are:
1. The causal bacteria are adhered to a surface.
2. Direct examination of infected tissue revealed bacterial colonies embedded in the matrix.
3. The infection is confined to one specific area.
4. Bacteria cannot be killed with systemically applied antibiotics even though their planktonic counterparts are highly susceptible to the same antibiotics.

5. Ineffective host defence demonstrated by bacteria surrounded with immune cells.

6. Elimination or disruption of biofilm structure leads to remission of the disease.

Apical periodontitis fulfills at least four out of six criteria as shown in the study of Ricucci & Siqueira (2010a) (criterion 1, 2, 3 and 5). Even though not assessed in this study, it is widely known that apical periodontitis cannot be successfully treated with antibiotics taken systemically as there is normally no circulation in a necrotic pulp that can bring the antibiotics (criterion 4) to the bacteria. Also, the existence of biofilms in treated canals and PTED suggest potential fulfillment of the criterion 6 (Siqueira et al. 2012).

Endodontic infections can be defined as infections of the pulp and periapical tissue. The difference between caries and periodontal infections and endodontic infections is that healthy pulp and periapical tissue are sterile and not exposed to oral bacteria. Even though diverse chemical and physical agents can cause irritation and sometimes necrosis of the pulp, bacteria and/or their products are the most frequent cause of pulpal inflammation (Watts & Paterson 1983, Seltzer & Farber 1994, Haapasalo et al. 2005). There are a number of possible pathways for bacteria to enter the pulp. These include carious lesions, enamel and dentin cracks, fractures, open dentinal tubules, lateral canals, invaginations, evaginations, leaky restorations and rarely also via anchoresis (Watts & Paterson 1983, Haapasalo et al. 2003). However, a carious lesion is one of the most common pathways causing endodontic infections. Bacteria from a carious lesion invade and multiply within dentinal tubules allowing access to the pulp chamber. Dentinal tubules range in size from 1
to 4 µm in diameter (Schilke et al. 2000), whereas the majority of bacteria are less than 1 µm in diameter. A tooth with a vital pulp is resistant to bacterial invasion. However, it has been shown that bacteria and their by-products have a direct effect on the dental pulp even without direct exposure (Bergenholtz & Lindhe 1975, Brännström et al. 1979, Bergenholtz et al. 1982, Cox et al. 1985). An inflammatory reaction in the pulp caused by an immune response of the body to bacterial antigens has been demonstrated (Bergenholtz 1990, Pashley 1996). However, as long as there is a healthy primary or secondary dentin wall separating the infection from the pulp, repair of the induced lesion usually takes place and pulp vitality is not normally jeopardized.

As the carious process advances, the pulp undergoes a variety of morphological and histological changes. The earliest pulpal changes in the odontoblastic layer precede inflammatory changes in the subodontoblastic region (Brännström & Lind 1965). Potential for pulp survival and repair seem to exist as long as infecting bacteria are not established in an attached biofilm layer in the pulp chamber. However, this has not been carefully examined. Histological sections have revealed a localized necrotic zone surrounded by a thin zone of hyperemia and accumulation of mainly polymorphonuclear cells. At a distance of 2-3mm from the necrosis and bacteria, the pulp tissue usually appears to be healthy (Trowbridge 1981). As long as the pulp is vital, there are limited numbers of bacteria in the pulp and therefore the prognosis of endodontic treatment of teeth with vital pulp is excellent (Engström & Lundberg 1965, Friedman 1998, Kojima et al. 2004). If untreated, the diseased area grows in size and the bacteria invade deeper into the root canal causing irreversible pulpitis and eventually pulpal necrosis (Seltzer et al. 1963a, 1963b, ABE 2007, Torabinejad & Walton 2008, Berman & Hartwell 2011). Necrotic pulp is rapidly invaded
and colonized by bacteria. Once the dental pulp is necrotic, the root canal system becomes “the source” of bacteria, bacterial by-products, and degradation products both from the bacteria and the pulpal tissue (Möller et al. 1981, Sundqvist 1992).

Apical periodontitis is an inflammation of the periradicular tissues caused by microorganisms in the necrotic root canal (Kakehashi et al. 1965). To heal apical periodontitis, microorganisms within the root canal system must be eradicated. Several studies have pointed to the importance of eliminating the microorganisms from the root canal and obtaining a negative culture at the time of obturation to achieve a better prognosis (Engström 1964, Katebzadeh et al. 2000). The elimination of microorganisms from the root canal is constrained by both the specific anatomy of the canal as well as the inability of host defenses to reach bacteria in the necrotic pulp tissue. Consequently, to eliminate an endodontic infection several host and treatment factors must be involved. If sufficient reduction of the infective microorganisms is attained, apical healing will be achieved.

1.3 Microbiology of Endodontic Infections

1.3.1 Normal Oral Flora

Before defining the microbiology of endodontic infections, normal bacterial flora of the oral cavity should be discussed. The mouth possesses a diverse but characteristic resident microbial community (Wilson 2005, Marsh & Martin 2009). Bacteria are the most numerous group and, initially, they were characterized solely using microscopy, cultural
techniques and biochemical tests. The recent application of molecular approaches (culture-independent methods) has provided deeper insights into the true complexity of the resident oral microflora. The oral microorganisms obtain their nutrients primarily from endogenous sources, such as amino acids, proteins and glycoproteins in saliva and gingival crevicular fluid; the metabolism of these substrates leads to only minor and slow changes to the local pH. Saliva also plays a major role in maintaining the oral pH at approximate neutrality, which is optimal for the growth of the majority of the microorganisms associated with oral health (Wilson 2005, Marsh & Martin 2009).

The composition of the oral microflora varies significantly at distinct surfaces within the mouth (e.g., tongue, buccal mucosa, and teeth) due to differences in key environmental conditions (Wilson 2005, Marsh & Martin 2009) emphasizing the link that exists between the properties of the habitat and the organisms that are able to establish themselves and then predominate.

Microbiological analysis of nine sites in the healthy oral cavity (dorsum of the tongue, lateral sides of the tongue, buccal epithelium, hard and soft palate, surfaces of the teeth, subgingival plaque, maxillary anterior vestibule, and tonsils) using the Polymerase Chain Reaction (PCR) technique found the average number of species in each site to be approximately twenty per person with the exception of the anterior vestibule which had up to nine species. Predominant bacterial flora included the phyla Firmicutes (Streptococcus, Gemella, Eubacterium, Selenomonas, Veillonella and related ones), Actinobacteria (Actinomyces, Atopobium, Rothia, and related ones), Proteobacteria (e.g. species of Neisseria, Eikenella, Campylobacter), Bacteroidetes (Porphyromonas, Prevotella,
Capnocytophaga), and Fusobacteria (e.g. species of Fusobacterium and Leptotrichia). Some species showed site specificity so that Streptococcus sanguis, S. gordonii, Abiotrophia defectiva and Actinomyces spp. were exclusively found on the tooth surfaces (Aas et al. 2005).

1.3.2 Carious Lesion

Bacteria associated with dental caries have been considered a major contributing factor leading to pulpal and periapical infections (Trowbridge 1981). There is substantial evidence that S. mutans is associated with the onset and development of dental decay (Edwardsson 1968, Loesche et al. 1975, Loesche 1986, Alaluusua et al. 1987). S. sobrinus has been implicated in caries development predominantly in instances where caries development appears to be independent of S. mutans. Interestingly, S. sobrinus displays higher acid production and acid tolerance than S. mutans. However, its presence in dental biofilm is more variable in biofilms than S. mutans. Other acidogenic and aciduric bacteria include Actinomyces spp. and Bifidobacterium spp. Traditional culture-based methods have shown that S. mutans is the chief pathogen for dental caries initiation (Loesche & Grenier 1976, Kolenbrander 2006, Wolff et al. 2012). However, growth of S. anginosus, S. constellatus, S. parasanguinis together with S. mutans on mitis salivarius-bacitracin (MSB) media formulations has been reported (Beighton 2005), thus complicating the interpretation of microbiologically derived evidence concerning S. mutans as the primary aetiological agent of caries development. While S. mutans has been found to be the predominant species in carious lesions in enamel, lesions in dentin harbour a variety of facultative and anaerobic bacteria including species of genera Lactobacillus, Actinomyces, Propionibacterium,
Bifidobacterium, Rothia, Eubacterium and Streptococcus (Edwardsson 1974). Gram negative anaerobic bacteria are also present in carious dentin (Hoshino 1985). Additional evidence has linked the Actinomyces to the onset and development of root surface caries (Van Houte 1994).

Relatively few studies have employed culture-independent techniques to study dental caries associated flora. 16S rDNA analysis have detected S. sanguinis and other species associated with dental health in caries-free children and adults, whereas Streptococcus spp. (S. mutans and low-pH non-S. mutans streptococci,) Veillonella spp., Actinomyces spp., Bifidobacterium spp., Lactobacillus spp., Propionibacterium spp., and Atopobium spp and other genera associated with dental caries both in primary and permanent dentitions have been demonstrated in caries-active respondents (Becker et al. 2002, Aas et al. 2008). The predominant microbes in a small number of adults with advancing, deep carious lesions were S. mutans and Lactobacillus spp. but included also species from the genera Prevotella, Selenomonas, Dialister, Fusobacterium, Bifidobacterium, and Pseudoramibacter (Munson et al. 2004, Chhour et al. 2005). However, the problem of dental decay is likely to be significantly more complex. It may be that many species in specific combinations will dictate dental health and disease or the transition between the two states (Peterson et al. 2011).

1.3.3 Root Canal Infection and Apical Periodontitis

Bacteria from non-treated carious lesions eventually reach the pulp. Although S. mutans and other streptococci may be involved in the initiation of caries, few of these species are
involved in the subsequent root canal infection, where the colonizing bacteria are of
different nature. The fate of bacteria entering the root canal is dependent on a variety of
factors. Bacterial survival depends on redox potential (the level of oxygen) in the root
canal, availability of nutrients, bacterial interaction as well as the host defense. Only
bacteria that can adapt to the ecological conditions of the canal can survive (Sundqvist
1992, 1994, Haapasalo et al. 2005). In primary endodontic infections, the amount of
oxygen in the root canal is low; therefore, anaerobic bacteria predominate often in
coexistence with microaerophilic and facultative Actinomyces spp. Lactobacillus spp. and
streptococci (Bergenholtz 1974, Fabricius et al. 1982). While a mixed anaerobic infection is
characteristic for primary endodontic infections, monoinfections (e.g. Enterococcus
faecalis) can be found in post-treatment cases of apical periodontitis (Siren et al. 1997,
Peculiene et al. 2000, Siqueira & Rôças 2004). Gram-negative enteric rods (e.g. coliforms
and Pseudomonas spp.) and yeasts are almost exclusively found in post-treatment apical
Furthermore, Chávez de Paz has reported gram positive flora predominating in apical
periodontitis following root canal treatment (Chávez de Paz et al. 2003).

Since host defense cannot effectively access and function in the necrotic root canal, bacteria
survive and can cause apical inflammation. Apical periodontitis is an inflammatory disease
with a microbial etiology (Sundqvist 1976, Moller et al. 1981). It is a response to intra-
radicular bacteria and comprises an effective barrier against spreading of the infection to
the alveolar bone and other body sites. In most situations, apical periodontitis lesions are
free of microorganisms. In primary endodontic infections, the main root canal contains the
majority of the flora. Bacteria from the main root canal can be treated by instrumentation
and irrigation during endodontic procedures. However, often bacteria will invade the
dentinal tubules and lateral canals as well as the fins and webs throughout the canal. In
about 50-80% of teeth with apical periodontitis, bacteria are found in the dentinal tubules
(Shovelton 1964, Nair 1987, Peters et al. 2001a). Resorption of root surface cementum is
an additional factor that facilitates bacterial penetration into dentinal tubules (Love 1996).
The most common invaders are non-motile bacteria; streptococci, enterococci, lactobacilli
and *Actinomyces* spp., and the greatest numbers of bacteria have been found in the coronal
and middle portions of the root canal wall (Peters et al. 1995).

In specific circumstances, the inflamed periapical tissue can be invaded by microorganisms
causing an extra-radicular infection. The most common form of extra-radicular infection is
acute periapical abscess, characterized by purulent inflammation in the periapical tissues in
response to the invasion of virulent bacteria from the root canal (Siqueira 2002). Osteomyelitis of the jaws arises primarily from odontogenic infection incited by one of two
major events: extension of a periapical tooth abscess or a posttraumatic or postsurgical
complication (Hudson 1993, Sharkawy 2007). Suppurative odontogenic infection,
including periapical abscess and osteomyelitis, is usually polymicrobial in nature, where
*Fusobacterium nucleatum*, pigmented *Prevotella*, *Peptostreptococcus*, *Actinomyces*, and
*Streptococcus* species are the predominant isolates (Tanner & Stillman 1993). There is
another form of extra-radicular infection that, unlike the acute abscess, is usually
characterized by the absence of evident clinical symptoms. This condition consists of the
establishment of microorganisms in the periapical tissues, either by their adherence to the
apical root surface in the form of biofilm-like structures (Tronstad et al. 1990) or within the
body of the inflammatory lesion, usually as cohesive colonies (Figdor 2002). Because
conservative endodontic treatment can not eliminate bacteria outside root canals such extraradicular microorganisms have been regarded as important in the pathogenesis of post-treatment endodontic disease (PTED) (Tronstad et al. 1990, Figdor 2002, Haapasalo et al. 2008).

1.3.4 Bacteria on External Root Surface

The traditional view has been that the root canal system and dentinal tubules harbour the infecting microorganisms, whereas the extraradicular area in an asymptomatic apical periodontitis lesion is free from bacteria because the host immune defense eliminates the bacteria that try to invade the periapex (Hornef et al. 2002). The presence of bacteria in the extraradicular area has been confirmed in post-treatment endodontic disease (Nair 1987, Sundqvist et al. 1998, Sunde et al. 2000, Cheng-Mei Yang et al. 2010, Su et al. 2010). In a recently reported case of PTED of a lower-left first molar that required apical surgery, the presence of a bacterial biofilm surrounding the apical foramen and external radicular surface was demonstrated with scanning electron microscope (SEM). Using culture techniques the following species were detected: *Actinomyces naeslundii* and *A. meyeri*, *Propionibacterium propionicum*, *Clostridium botulinum*, *Parvimonas micra*, and *Bacteroides ureolyticus* (Signoretti et al. 2011). Similarly, SEM identified microbial biofilms made up mostly of cocci and rods in scattered areas of the root surface in eight extracted teeth with persistent periradicular lesions that had not responded to previous root canal treatment (Leonardo et al. 2002). Calculus-like deposits (most probably calcified biofilms) on the external root surface of a tooth with PTED has been described by Ricucci et al. 2005. Using a PCR based 16S rRNA gene assay, 31 bacterial species [majority
(51.6%) of the strains were facultative anaerobes] from 20 apical lesion samples with persistent periapical infection have been detected (Fujii et al. 2009). The predominant genera were *Staphylococcus, Propionibacterium, Prevotella, Streptococcus, Fusobacterium,* and *Pseudomonas.* Similarly, 16S rRNA analysis of the root canals of 33 root-filled teeth with persistent periapical infections referred for surgical treatment detected *Porphyromonas endodontalis, P. gingivalis, A. viscosus,* and *Candida albicans* as the dominating species (Zhang et al. 2010). However, the possibility of contamination of the surgical site during the operation from plaque, mucosal or even salivary microorganisms is very difficult to completely rule out (Sunde et al. 2000). Therefore, the results of these studies should be evaluated with caution.

1.3.5 Extraradicular Infection

In the majority of teeth associated with apical periodontitis, infection is restricted to the root canals. Most of the microbial species that infect the root canal (Siqueira 2002) do not have the ability to survive host defence mechanisms in the periapical tissues. However, microorganisms that possess strategies to survive and infect vital tissues must be able to scavenge nutrients and escape the host defence mechanisms (Siqueira 2002). In these cases, an extra-radicular infection may develop. Several species of documented oral pathogens have been detected in post treatment apical periodontitis lesions (Tronstad et al. 1987, Gatti et al. 2000, Sunde et al. 2000). Some of them possess virulence mechanisms that theoretically can allow them to invade and to survive in a hostile environment (Siqueira 2002). However, their involvement in an extra-radicular infectious process independent of the intra-radicular infection is not certain (Sunde et al. 2000, Haapasalo et al. 2008).
Even though *Actinomyces* spp. have been commonly isolated from periapical lesions in PTED, many studies have indicated that bacteria other than *Actinomyces* spp. can form actinomycosis-like colonies in the periapical area (Haapasalo et al. 2008). Bacteria that can cause extraradicular infection are normal inhabitants of the oral cavity (Slack & Gerencser 1975, Bowden 1998, Smego & Foglia 1998) and can enter the periapical area from the root canal either through lateral canals or dentinal tubules (Tronstad et al. 1990). Also, bacterial colonies in the form of biofilms can be transferred into the periapical lesion during root canal instrumentation (Haapasalo et al. 2008). There is also the possibility that contact of the teeth during occlusal activity can cause liquid movement through the tooth's apex and consequent bacterial transfer to the periapical tissue (Kishen 2005). In addition, a long-standing sinus tract infection can act as possible pathway for bacteria to enter periapical lesion (Ricucci et al. 2005). Actinomycosis, the most commonly described extraradicular infection is a chronic, granulomatous infectious disease characterized by suppuration, abscess formation and draining sinus tracts, which erupt to the skin or mucosal surfaces and drain pus containing “sulfur granules” (small colonies of bacteria) (Slack & Gerencser 1975, Smego & Foglia 1998). The basic microscopic picture in actinomycosis is suppurative, but it can vary from an acute abscess to a chronic lesion in which proliferating connective tissue is commonly seen (Slack & Gerencser 1975). Periapical actinomycosis as an extra-radicular infection can be independent of the bacteriological status of the root canal of the affected tooth. The causative bacteria may invade the periapical tissues and establish equilibrium with the host without inducing acute inflammation with clear symptoms. The treatment of periapical actinomycosis includes periapical surgery or tooth extraction.
1.4 Biofilms

1.4.1 A Brief History of Biofilms

The first evidence of biofilms was given by the Dutch scientist Anthony van Leeuwenhoek over 300 years ago. He scraped material from his own teeth and using simple microscopes observed a great number of moving objects not visible by the naked eye. Since he thought they were tiny living animals, he reported them as *animalcules* (Dobell 1958). Even though he was not aware of the importance of his achievement, that observation made him the first biofilm experimenter. In 1933 Arthur Henrici, an American bacteriologist, noticed a growth of algae on the walls of an aquarium in his laboratory (Henrici 1933). In order to study this growth he placed microscope slides in his aquarium. However, he noticed that besides the algae, various bacteria were thinly coated on the slides. Therefore, he assumed that most water bacteria do not float freely but rather they grow on submerged surfaces (Henrici 1933). Finally, Claude Zobell, a microbial ecologist, reported that when seawater was collected in sterile glass bottles, more bacteria were later present on the surface of the glass than in the seawater. That was another confirmation of the growth of sessile bacteria (Zobell 1937, 1943).

These pioneering scientists all described what we now call biofilm. Biofilms are complex microbial communities attached to living or non-living surfaces and composed of a great variety of bacteria embedded in a self-produced, highly hydrated matrix of an extracellular polymeric substance (EPS) (Allison et al. 2003). Even though they can be present in various environments and may have some differences with regard to their structure,
composition and activity, biofilms generally appear similar and the processes for their establishment on the surface are most probably related (Svensäter & Bergenholtz 2004). Numerous *in vitro* and *in vivo* studies on dental plaque, an example of biofilm, have greatly contributed to our knowledge about these communities (Bowden & Hamilton 1998, Marsh 2004).

1.4.2 Biofilm Formation

The formation of microbial (dental) biofilm is a dynamic event which includes five successive steps: reversible attachment, irreversible attachment, development of microcolonies, maturation and dispersion (Dufour et al. 2012) (Figure 1). In the first step, bacteria attach to the selected abiotic or biotic surface. Within moments of placing a clean sterile slide into a water source, stream, aquarium or on professionally cleaned tooth surfaces, a film that consists of proteins and polysaccharide molecules begins to form. This conditioning film is typically composed of organic molecules (e.g. nutrients, salivary proteins and glycoproteins, large macromolecules) that can help the bacteria adhere to the surface (Dufour et al. 2012). Weak reversible van der Waals interactions between the cell surface and the substratum are responsible for the initial bacterial attachment leading to stronger adhesin-receptor mediated attachment (Donlan 2002). Flagella, fimbriae, lipopolysaccharide (LPS), exopolysaccharides and other bacterial cell-surface structures play a role in irreversible interactions. The second step involves the aggregation and the third step includes development of microcolonies promoted by the growth and the division of the first attached cells (primary colonizers). It has been shown that specific species are considered to be early colonizers that facilitate the adhesion of bacteria in the later stage of
biofilm formation. For example, some early colonizers, such as streptococci, affect later adhesion of both Gram-positive and Gram-negative bacteria (Kolenbrander et al. 2002). The number of bacteria progressively increases and unites to form a first layer of cells covering the surface (Dufour et al. 2012). In the fourth step, after multiplication of the attached bacteria, biofilm becomes a structurally organized microbial community embedded in extracellular polymeric substance. The observation of mature biofilms using confocal laser scanning microscope has revealed micro-colonies embedded in the matrix with open water channels lined with the most viable bacteria (Netuschil et al. 1998). To spread and colonize other niches, some biofilm cells detach individually or in clumps during the fifth step. In general, biofilm dispersion occurs in response to environmental changes and reflects biofilm growing conditions (O’Toole et al. 2000).

Both genetics (active response) and environmental conditions (passive response) contribute to the development of biofilm (Hall-Stoodley et al. 2004). Even though numerous genes have been identified as “essential” for biofilm formation, it has been shown that “knocking out” of these specific genes only slows down the formation of biofilms (Hall-Stoodley et al. 2004). On the other hand, the environmental conditions that affect biofilm development are temperature, pH, O₂ levels, hydrodynamics, osmolarity, the presence of specific ions, nutrients, and factors derived from the biotic environment (Goller & Romeo 2008). Nutrition availability is a limiting factor for biofilm growth as observed by Heydorn et al. (2000) who found increased thickness of Pseudomonas aureofaciens biofilms after the addition of increasing concentrations of citrate. Similarly, Chávez de Paz (2012) reported continuous biofilm growth in rich medium over time contrary to smaller biovolumes in the
absence of glucose. However, most of the cells remained viable even during the starvation phase.

Biofilms display a common attribute, the matrix, or extracellular polymeric substance. This self-produced extracellular matrix holds bacteria together in these complex communities (Flemming & Wingender 2010). Biofilms are composed of about 80-85% EPS (by volume) and only 15-20% cells (volume). EPS may vary in chemical and physical properties in various biofilms; however, its major components are polysaccharides (homo- and
heteropolysaccharides), proteins, and extracellular DNA. Highly hydrated EPS prevents desiccation in some natural biofilms and also acts as a diffusion barrier preventing toxic substances such as antibiotics and disinfectants from reaching their target, the bacterial cells (Dufour et al. 2012).

Most biofilms found in nature are polymicrobial, comprising a multitude of different species expressing multiple phenotypes. Human dental plaque biofilm and the biofilms associated with periodontal and endodontic diseases are probably the best described multi-species microbial biofilms. However, even in a monospecies biofilm, phenotypic heterogeneity exists. It is possible that cells of the same bacterial species can exhibit different phenotypes in a biofilm even though they are separated by as little as 10 μm (Socransky & Haffajee 2002). Cell differentiation in biofilms may be regulated by the local environmental conditions surrounding the cells such as different concentration gradients of oxygen, nutrients, ions, and other chemicals. One good example of phenotypic heterogeneity is given in the study of Stewart & Franklin 2008. In a mono-species biofilm of a facultative anaerobic bacterial species, individual bacterial cells responded to the local microenvironment manifesting phenotypic heterogeneity. Cells in the upper biofilm layers grow aerobically and consume all available oxygen. This makes it possible for an anaerobic micro-niche to develop under the aerobic layer. Oxygen- and nutrient depleted regions are found at the bottom layers of the biofilm structure and under these circumstances, most of the cells are metabolically inactive or dead (Stewart & Franklin 2008). Phenotypic heterogeneity may occur spontaneously, as a result of genetic mutations or through adaptive mutations. The latter encourages survival of the population as a whole under stress conditions (Plakunov et al. 2010). Bacteria in biofilms express phenotypes which are
different from their planktonic counterparts. Sauer et al. (2002) characterized five distinct stages in *P. aeruginosa* PAO1 biofilm formation by examining changes in protein and gene expression over a 12-day period. His study suggested expression of multiple phenotypes over time, and after 12 days of biofilm growth only 40% of the expressed proteins were similar to their planktonic counterpart.

### 1.4.3 Communication Language in Biofilm

Quorum sensing was first discovered in 1970 in a gram negative bioluminescent bacterium *Vibrio fischeri*. This organism lives in the photophore (or light-producing organ) of the Hawaiian bobtail squid as a mutualistic symbiont (Nealson et al. 1970). When *V. fischeri* cells are free-floating (planktonic), the cells do not luminesce. However, when they are highly concentrated in the photophore (about $10^{11}$ cells/ml), transcription of luciferase is induced, leading to bioluminescence (Dunlap 1999). Therefore, quorum sensing can be defined as a system of stimulus and response correlated to population density. In other words, it is a special communication language between bacteria that becomes significant after the biofilm reaches a critical number of cells (Ng & Bassler 2009). This cell to cell signaling system is carried out by signal molecules synthesized intracellularly and released passively or actively into the surrounding environment. These signal molecules, termed autoinducers, increase in concentration as a function of bacterial cell density. When bacteria reach critical mass, the corresponding levels of signaling molecules induce a synchronized response in gene expression throughout the bacterial population (Li & Nair 2012).
Quorum sensing signal molecules include three major types of autoinducers. These are acyl homoserine lactone (AHL) in Gram negative bacteria (Manefield & Turner 2002), oligopeptides in Gram positive bacteria (Mayville et al. 1999) and a class of autoinducer-2 (AI-2) in both Gram negative and positive bacteria (Schauder et al. 2001). AHL molecules are primarily associated with intraspecies communication in Gram negative bacteria where unique molecule of AHL can be recognised only by the members of the same species. Four key components are recognised in AHL signal system. These are AHL synthase (lux I type), AHL signal molecule, luxR type receptor and target genes (Hentzer & Givskov 2003). This signaling system has been best described in P. aeruginosa (Müh et al. 2006). Autoinducing peptides (AIPs) used as signaling molecules in Gram positive bacteria are modified oligopeptides typically consisting of 5 to 17 amino acids (Lazazzera & Grossman 1998). Their mechanism of action includes inducing a cascade of the phosphorylation of regulator proteins allowing the signal to regulate gene expression (Novick 2003). For inter-species communication, major bacteria (Gram positive and Gram negative) use AI-2 (Chen et al. 2002) Synthesis of AI-2 molecule requires luxS synthase. To date it is the only known species-nonspecific autoinducer reported and it has been proposed as a universal signal for inter-species communication (Surrete & Bassler 1998, Miller & Bassler 2001, Schauder & Bassler 2001). Despite AI-2 being produced by many genera, there is very little evidence linking AI-2 with direct activation of any specific genes (Diggle et al. 2007).

Quorum sensing system plays an essential role in key bacterial behavior including virulence, biofilm formation and maturation, bacterial swarming, antibiotic production and
antibiotic resistance (Whitehead et al. 2001, Li & Nair 2012). The role of quorum sensing system in the regulation of biofilm formation was originally reported for *P. aeruginosa* (Davies et al. 1998). AI-2 is an important molecule in multispecies biofilm formation and maturation (Huang & Gregory 2011). Also, it mediates the formation of a mixed biofilm of oral Streptococci and Actinomyces (Rickard et al. 2006).

1.4.4 Biofilm Resistance to Antibacterial Agents

Bacteria organized in biofilms benefit from their community in various ways. For example, some bacteria do not possess enzymes to degrade large nutrient molecules and cannot survive in monoculture. However, in mixed biofilms they can survive in cooperation with other bacteria (Marsh et al. 2000). This may be one of the reasons why bacteria can survive periods of starvation and recover once nutrients become available (Svensäter & Bergenholtz 2004, Shen et al. 2010a). Increased virulence and resistance to antimicrobial compounds is another feature of bacterial interactions in the biofilm community (Fletcher 1991, Goodman et al. 1995).

One of the important characteristics of microbial biofilms is their high-level of drug tolerance. Perhaps the first experiment showing that biofilm cells are more tolerant to drugs than planktonic cells was done by Leeuwenhoek. He reported a failure to kill plaque bacteria on his teeth by prolonged rinsing with vinegar, whereas all the microorganisms were killed when he removed them from his teeth and mixed them with vinegar in the laboratory (Dufour et al. 2012). Up to 1000-fold higher concentrations of antimicrobials/antibiotics are needed to kill biofilm bacteria in comparison to their free-
floating counterparts (Davies 2003). Although antibiotics may reduce the amount of bacteria within biofilms, they cannot completely eradicate them and so refractory/relapsing infection may occur. A further challenge to treating biofilm infections is that the bacteria in biofilms exist in a protected mode of growth allowing the microbes to survive the host immune defence. Additionally, multiple factors contribute to the resistance of microbial biofilms to antibacterial agents (Stewart & Costerton 2001). Four mechanisms have been proposed to explain the reduced susceptibility of bacteria to antibacterial agents. These are a reduced penetration of agents imparted by the EPS matrix, the alteration of the microenvironment within the biofilm, the induction of specific resistance mechanisms and/or the presence of phenotypic variants or persister cells (Stewart & Costerton 2001). It is thus essential to understand the mechanisms that promote tolerance to antimicrobials to develop novel strategies to treat biofilm infections.

1.4.4.1 The Role of EPS in Biofilm Resistance

A primary function attributed to the EPS matrix is protection. This matrix protects the bacteria from the host defense and antibiotics. Several studies have shown that the EPS matrix can act as an impermeable barrier to limit antimicrobial penetration, thereby protecting the biofilm cells (Donlan 2002, Sutherland 2001). Such protection can be due to either physical interference in antimicrobial diffusion or direct binding of antibiotics by the EPS matrix. The biofilm matrix can also be considered a chemically active barrier. This mechanism may apply to reactive (superoxide and sodium hypochlorite), charged (metals) or large (immunoglobulin) antibacterial agents that are often diluted or neutralized below the toxic concentrations by EPS before they reach the individual bacterial cell (Hall-
It has been shown that a negatively charged EPS produced by *P. aeruginosa* binds to positively charged antibiotics such as aminoglycosides even though the resulting reduction in the diffusion coefficient cannot explain the observed changes in the susceptibility of the biofilm (Nichols et al. 1988). Costerton (1999) reported that β-lactam antibiotics can penetrate the entire thickness of the biofilm and concluded that protection of biofilm cells is not really linked to EPS.

1.4.4.2 Alteration of Microenvironment within the Biofilm

It is well known that nutrients are not equally available in all parts of biofilms. Also, oxygen level can vary throughout the biofilm where it can be completely consumed from the surface area leading to formation of anaerobic niches in deeper layers (de Beer et al. 1994). Similarly, acidic waste products might lead to differences in pH in some biofilm areas (Zhang & Bishop 1996). These conditions can slow down or antagonise the action of an antibiotic. For example, aminoglycoside antibiotics are less efficacious against bacteria in aerobic than in anaerobic conditions (Tack & Sabath 1985). In addition, microbial cells localised in deeper layers of biofilms (areas of limited nutrients and oxygen), have reduced metabolic activity that might account for reduced sensitivity towards antibiotics that target bacterial cellular processes in the period of cell division (Tuomannen et al. 1986).

1.4.4.3 Special Mechanisms of Biofilm Resistance

One recently explored mechanism of bacterial resistance to antibiotics is the drug efflux pump. This mechanism actively exports the antibiotic from the cell (Piddock 2006). Drug
efflux pumps have been found both in Gram negative and Gram positive bacteria (Li & Nikaido 2004). There has been evidence that they are induced by exposure to sub-lethal concentrations of various antibiotics (Lynch & Robertson 2008). The primary role of efflux pumps is to export several unrelated substances, including molecules produced by the host organism (such as bile), indicating that multidrug mediated resistance is a by-product of the physiological role(s) of these pumps (Piddock 2006). Efflux pumps can show specificity for a single substrate, or they can transport a range of dissimilar compounds (including different chemical classes of antibiotics) (Piddock 2006).

The role of quorum sensing and intracellular signalling molecules in antimicrobial tolerance of biofilms has also been investigated in several studies (Bjarnsholt et al. 2005, Möker et al. 2010). “Quorum quenching” is a new strategy for elimination of biofilm bacteria based on interference with cell-to-cell communication and signalling molecules (Dong et al. 2007). It specifically targets the quorum sensing cascade without affecting bacterial viability and growth (Bandara et al. 2012). Quorum sensing inhibitors are promising treatment alternatives to antibiotics whose use is limited due to increased antibacterial resistance (Uroz et al. 2009). It has been demonstrated that quorum sensing inhibitors enhanced the susceptibility of \textit{P. aeruginosa} biofilms to antimicrobial treatments (Bjarnsholt et al. 2005). Three main strategies can be used in quorum quenching: prevention of signal generation, dissemination, or perception (Hentzer & Givskov 2003, Uroz et al. 2009).
1.4.4.4 Persister Cells

Persister cells represent another mechanism of antimicrobial resistance. Small subpopulations of non-growing bacterial cells (0.001% to 0.1% of the total population) that exist within any population of bacteria are referred to as persister cells (Lewis 2010, Jayaraman 2008). Persister cells are described as specialized cells that enter into a dormancy state, which enables them to survive stress conditions and prevents the death caused by antimicrobials (antibiotics) that target cell growth (Bigger 1944). In contrast to resistant cells, persisters are genetically identical to the susceptible bacteria representing phenotypic variants (Kint et al. 2012). Persisters exist both in planktonic and biofilm culture, however, they present a small fraction of free-floating cells while they are numerous in biofilms. Persister cells are also growth-phase dependent; their number is low in exponentially growing cultures and their number reaches a maximum when the cells are in their stationary phase (Keren et al. 2004). The importance of persister cells can be illustrated by comparing antibiotic treatment of planktonic cells and biofilm bacteria. A mechanism for the antibiotic resistance of persister cells has been proposed by Lewis in 2008. Antibiotic treatment easily kills most planktonic bacteria except for the persisters which are targeted by the host immune system. The situation is different in biofilms because the biofilm persisters are protected from the host defense by the biofilm matrix. Soon after a course of antibiotic treatment is completed, the protected persister cells repopulate the whole biofilm population (Lewis 2008). Therefore, persisters may have greater clinical significance in biofilm infections, especially in immunocompromised patients (e.g. HIV-positive, cancer, chemotherapy) (Lewis 2010).
Why persister cells are more resistant to antimicrobial agents is not known with certainty. There are several proposed mechanisms for the formation of persister cells (Kint et al. 2012). One of them includes active induction of oxidative stress mechanisms which lowers the cellular reactive oxygen species (ROS) levels induced by bactericidal antibiotics. Also, DNA-damaging antibiotics can induce persister cells by triggering a response for DNA repair. Sporadic expression of toxic proteins in a small fraction of cells, which maintains them in a dormant or non-growing state, can be another mechanism of persisters’ formation (Kint et al. 2012). These toxic proteins are small genetically-encoded modules containing two components: a stable toxin and its labile antitoxin. Usually, the toxin inhibits an essential microbial cell function such as translation or DNA replication (Haynes 2003). It has been also shown that ectopic mild-overexpression of unrelated proteins also induces persistence (Lewis 2010). Finally, chemical signaling may play a role in the induction of persistence either through chemical signaling from nutrient limitation or by quorum sensing related signaling molecules (Kint et al. 2012).

1.4.5 Biofilms in the Root Canal

Biofilms in the oral cavity exist in health but under specific conditions they can also cause different diseases such as caries, gingivitis, periodontitis and endodontic infections. The development of caries, gingivitis, periodontitis and acute exacerbations of endodontic infections is considered a consequence of disrupted ecological balance in biofilms caused by availability of nutrients, change in the pH or any other specific condition which favors the growth of some strains important for the evolution of a specific disease (Svensäter & Bergenholtz 2004).
The first evidence of biofilms in root canals was presented by Nair (1987). After examination of the root canal contents of 31 teeth using transmission electron microscopy, he observed both “loose” bacteria and dense microbial aggregates with an amorphous substance which he interpreted as an extracellular matrix. Several other studies have revealed the presence of micro-colonies of cocci and rods on the wall of the root canal even though a typical biofilm structure was not seen (Molven et al. 1991, Sen et al. 1995). Tronstad et al. (1990) described biofilms on the external surface of the root apex by observing root tips removed during periapical surgery. This finding was confirmed by other studies (Leonardo et al. 2002, Ricucci et al. 2005, Wang et al. 2012a). However, the recognition of periapical abscess as a source for extra oral presence of bacteria has not been properly underlined (Siqueira & Lopes 2001). Based on their observations, it has been considered that in primary periapical infections bacteria are not commonly found outside the root canal space. On the other hand, in post-treatment endodontic infections and resistant periapical infections, bacteria can be found on the external surface or the root apex (Siqueira 2000, Noiri et al. 2002, Sunde et al. 2002, Ricucci et al. 2005).

1.5 Endodontic Treatment

The goal of endodontic treatment is to prevent or eliminate apical periodontitis (Pitt Ford 1982, Ørstavik & Pitt Ford 1998, Trope et al. 1999, Ørstavik et al. 2004). To achieve this goal, bacteria should either be prevented from entering the root canal (prevention of AP) or eliminated from the root canal. Chemomechanical instrumentation has been regarded as the key element of endodontic treatment. With instrumentation, vital and necrotic organic tissue as well as some hard tissue is removed and adequate space for irrigation and/or
medication and eventually obturation is created. However, it has been shown that all bacteria cannot be eliminated from the root canal by instrumentation only (Byström & Sundqvist 1981, Ørstavik & Pitt Ford 1998, Pataky et al. 2002). In addition to mechanical preparation, irrigating solutions with a strong antibacterial effect are necessary. Currently, commonly recommended irrigation protocols include the use of sodium hypochlorite (NaOCl, 0.5-6%) followed by ethylenediaminetetraacetic acid (EDTA; 17%). Chlorhexidine [(CHX) 2%] has also been used as a final rinse after EDTA (Zamany et al. 2003). Sodium hypochlorite has a strong antibacterial effect and dissolves necrotic organic tissue (Rosenfeld et al. 1978), while EDTA acts on inorganic tissue and removes the smear layer (Czonstkowsky et al. 1990) after the use of NaOCl. CHX is an antibacterial agent that can kill bacteria directly and bind to the teeth and mucous membrane where it is released during an eight to twelve hour period (Stabholz et al. 1993, White et al. 1997, Gomes et al. 2001, Vianna et al. 2004). However, none of these available irrigants alone is capable of eliminating all bacteria from the root canal (Haapasalo et al. 2010). Therefore, the search for more effective irrigants is ongoing.

1.6 The Statement of the Problem

1.6.1 Background

Bacteria in nature rarely exist as freely floating (planktonic) organisms. However, the majority of endodontic studies testing the antibacterial efficacy of antibiotics or other disinfecting agents have been performed on bacteria in planktonic culture (Shih et al. 1970, Barnard et al. 1996, D'Arcangelo et al. 1999, Steinberg et al. 1999, Gomes et al. 2001,
Torabinejad et al. 2003a, Portenier et al. 2005). Furthermore, it has been shown that although most of the antibacterial agents are capable of rapidly killing planktonic bacteria, this does not correlate with the antibacterial efficacy of the same agents when treating infections \textit{in vivo}. Biofilms represent the most common form of bacterial existence in nature and are the main cause of chronic infections. Because of the discrepancy between the ability of antibacterial agents to kill bacteria in planktonic phase and to kill bacteria in biofilms, it is important to focus on biofilms when evaluating these agents. Biofilm bacteria can be up to 1,000 times less sensitive to antibacterial agents (Ceri et al. 1999) than their planktonic counterparts. Studies on \textit{in vitro} biofilms have used a variety of biofilm models to evaluate the role of variables such as composition, age, substrate and nutrient availability. Since \textit{E. faecalis} is an important pathogen in endodontic infections (especially in PTED), it has been the most commonly used species for studying single species biofilms in endodontics. Biofilms have been grown from 1 - 7 days (Duggan & Sedgley 2007, Brändle et al. 2008, Chávez de Paz et al. 2010) up to 42-160 days (Distel et al. 2002, Kishen et al. 2006, Estrela et al. 2009a). Microscope slides (Williamson et al. 2009), porcelain (Dunavant et al. 2006), polystyrene microtiter plates (Duggan & Sedgley 2007), nitro-cellulose membranes (Chai et al. 2007), dentin (Brändle et al. 2008) and hydroxyapatite (HA) discs (Shen et al. 2009, Deng et al. 2009) have been used as biofilm substrates. Biofilms have been grown either in aerobic (Duggan & Sedgley 2007) or anaerobic conditions (Brändle et al. 2008, Shen et al. 2009). Also, nutrient requirements have been addressed with different frequencies, i.e. every 2 to 5 days (Kishen et al. 2006, Brändle et al. 2008) or every 7-30 days (Shen et al. 2009). However, there is no standardised biofilm model to study biofilms or to simulate the characteristics and behavior of \textit{in vivo} biofilms.
1.6.2 Hypothesis

The central hypothesis of this thesis was that by using mixed bacterial flora from the oral cavity, it is possible to develop *in vitro* biofilms which share certain key features of *in vivo* biofilms. Such standardised biofilms would allow evaluation of the antimicrobial properties of a wide variety of disinfecting agents and make it possible to compare the results of different experiments.

1. In Chapter 2 we hypothesized that the new *in vitro* biofilm model shares key characteristics with *in vivo* oral biofilms (as described in the literature) and that susceptibility of biofilm bacteria to antibacterial agents depends more on the level of biofilm maturation than on the source of biofilm bacteria, or the type of disinfecting agent.

The first objective was to study the structure of our (Shen et al. 2009) *in vitro* biofilm model in comparison with *in vivo* oral biofilms as described in the literature using transmission electron microscopy (TEM). Further, objectives were to assess the effect of the source of biofilm bacteria, the level of biofilm maturation, and the type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents.

2. In Chapter 3 we hypothesized that modified photoactivated disinfection (PAD) would be more effective against *E. faecalis* and mixed plaque bacteria, both in planktonic culture and in biofilms, than conventional PAD or the commonly used endodontic disinfecting agents used separately.
Our objective was to compare the efficacy of conventional and modified photoactivated disinfection against *E. faecalis* and mixed plaque bacteria in planktonic and biofilm culture.

3. In Chapter 4 we hypothesized that a new, multicomponent endodontic irrigant, QMiX, would be more effective than traditional endodontic irrigants (chlorhexidine, sodium hypochlorite) and another combination product, BioPure MTAD, against *E. faecalis* and mixed plaque bacteria in both planktonic and biofilm culture.

The objective was to assess, using an experimental laboratory model, the antibacterial efficacy of a novel root canal irrigant, QMiX, against *E. faecalis* and mixed plaque bacteria both in planktonic phase and in biofilms.

1.6.3 Rationale for the Study

There are no widely used, standardized biofilm models for the study of bacterial susceptibility in oral infections. Therefore, it has been difficult or impossible to compare the results from different studies or to interpolate them into the clinical situation. A variety of biofilm models have been used in studies of the antibacterial efficacy of disinfecting agents. Unfortunately, any long-term value of these studies has been limited as the models have often been poorly characterized. Therefore, one of the main goals of the present thesis was to develop a multispecies *in vitro* biofilm model having many similarities with true, *in vivo* biofilms as described in the literature. Furthermore, the documented difficulties in eliminating bacteria from infected root canals using current treatment protocols have
directed our research to modify and improve available disinfecting agents and/or protocols to achieve more predictable elimination of bacteria.
Chapter 2: Effect of the Source of Biofilm Bacteria, Level of Biofilm Maturation, and Type of Disinfecting Agent on the Susceptibility of Biofilm Bacteria to Antibacterial Agents

1 A version of this chapter was published. Stojicic S, Shen Y, Haapasalo M. Effect of the source of biofilm bacteria, level of biofilm maturation, and type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. J Endod 2013;39:473-7.
2.1 Introduction

Bacterial etiology has been confirmed for common oral diseases such as caries (Miller 1894) and periodontal (Slots 1979) and endodontic infections (Kakehashi et al. 1965). Bacteria causing these diseases are organized in biofilm structures, which are complex microbial communities, composed of a great variety of bacteria with different ecological requirements and pathogenetic potential. The biofilm community gives bacteria effective protection not only against the host’s defense system, but makes them also more resistant to different disinfecting agents used as oral hygiene products or in the treatment of infections (Stewart et al. 2001). Successful treatment of these diseases depends on biofilm removal as well as effective killing of biofilm bacteria. Since bacteria causing endodonic infections are mostly found in the main root canal, chemomechanical debridement plays a key role in treating endodontic infections. However, due to the complexity of root canal anatomy, it has been shown that about 35% of instrumented root canal area is left untouched when conventional rotary and hand instruments are used (Peters et al. 2001b). Therefore, elimination and killing of biofilm bacteria from the root canals relies to a great extent on the efficacy of endodontic irrigants and medicaments.

Although bacteria in nature rarely exist in planktonic phase, most of the studies of endodontic disinfecting agents have been done using bacteria in planktonic culture (Shih et al. 1970, Barnard et al. 1996, D'Arcangelo et al. 1999, Steinberg et al. 1999, Gomes et al. 2001, Torabinejad et al. 2003a, Portenier et al. 2005). However, it has been recognized that rapid killing of planktonic bacteria by various disinfecting agents does not reflect the effect of the same agent on bacteria in in vivo biofilms. It has been demonstrated that biofilm
bacteria can be 100 to 1,000 times more resistant to antibacterial agents (Ceri et al. 1999) than their planktonic counterparts. Because of this great difference, a growing number of studies now focus on the killing biofilm bacteria instead of planktonic bacteria by the disinfecting agents (Barrieshi et al. 1997, Dunavant et al. 2006, Kishen et al. 2006, Shen et al. 2009, 2010a, 2010b, 2011).

Biofilm growth is a continuous process initiated by the attachment of planktonic bacteria to a surface (substrate) and then proceeding through different stages into a mature, structurally complex biofilm (Sauer et al. 2002). To study oral biofilms, including root canal biofilms, it is important to develop multispecies in vitro biofilm models with close similarity to true in vivo biofilms. Analysis of such in vitro biofilms should provide information about biofilm characteristics that will eventually lead to finding better strategies for their elimination in vivo. A variety of different biofilm models have been used to evaluate the effectiveness of endodontic irrigants. Since there is no consensus on which specific biofilm model to utilize, the results from different studies cannot be easily compared or related to real clinical situations. Biofilms have been grown on different substrates (Clegg et al. 2006, Dunavant et al. 2006, Shen et al. 2009, Williamson et al. 2009) and for different time periods (Clegg et al. 2006, Dunavant et al. 2006, Shen et al. 2009, Williamson et al. 2009) in a variety of different growth conditions including the amount of oxygen (Distel et al. 2002, Brändle et al. 2008, Shen et al. 2009) and nutrient supply (Clegg et al. 2006, Brändle et al. 2008, Shen et al. 2009). In most studies the biofilms have been grown for 1 – 7 days only (Clegg et al. 2006, Dunavant et al. 2006, Williamson et al. 2009), while only occasionally longer times up to several months have been used (Kishen et al. 2006, Shen et al. 2009).
A new *in vitro* multispecies biofilm model that closely mimics the *in vivo* biofilm for evaluating the efficacy of endodontic irrigants has recently been introduced by Shen et al. (2009). Collagen-coated hydroxyapatite (CHA) discs provided the substrate for biofilm growth. A consistent biofilm thickness of about 150 µm was established across the HA surface. Beside cocci, rods, and filaments, spirochetes were found in high numbers throughout the biofilm surface (Shen et al. 2009). However, further analysis of this biofilm model has not been done yet.

The importance of biofilm age and nutrition on biofilm behaviour was demonstrated by Shen et al. (2011) who challenged young and old biofilms (from two days to 12 weeks) with two different chlorhexidine preparations for 1, 3 and 10 min. Results from this study showed that biofilms which were two weeks old or younger were much more sensitive to the tested agents than biofilms grown for three weeks or more. However, as all the biofilms in that study were grown from plaque bacteria obtained from one donor and only CHX products were tested, it is not possible to know whether the results can be generalized or if they represent the behaviour of that particular biofilm exposed to chlorhexidine only.

Therefore, the aim of this study was to 1) evaluate bacterial morphotypes and their associations in a Shen et al. (2009) *in vitro* multispecies biofilm model using transmission electron microscopy and 2) assess the effect of the source of biofilm bacteria, the level of biofilm maturation, and the type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents.
2.2 Materials and Methods

Sterile hydroxyapatite discs (9.6 mm diameter by 1.5 mm thickness; Clarkson Chromatography Products, Williamsport, PA, USA) were used as substrate to grow biofilms. The HA discs were coated with bovine dermal type I collagen (10 mg/ml collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA, USA). Collagen coating of the HA discs was done by overnight incubation at 4°C in the wells of a 24-well tissue culture plate (Costar, Corning, NY, USA) containing 2 ml of the collagen solution. Supragingival and subgingival plaque was collected from six adult volunteers, 25-45 years old with healthy gingiva, mild gingivitis or periodontitis and suspended in Brain Heart Infusion broth (BHI; Becton Dickinson, Sparks, MD, USA). Ethical approval for the study was obtained from UBC Research Ethics Board (the certificate number H12-00907). A separate batch of biofilms was grown from the plaque of each donor. CHA discs were placed in the wells of a 24-well tissue culture plate containing 1.8 ml of BHI and 0.2 ml of plaque suspension was added to each well. The discs were incubated at 37°C in BHI under anaerobic conditions (AnaeroGen, OXOID, Hampshire, UK) for 2 days, 1, 2, 3, 4 and 8 weeks. The BHI medium was changed once per week (from one to four weeks) and after four weeks it was not changed. Limiting the supply of fresh nutrients to the biofilms was based on the following reasons: 1) access to nutrients in the root canal is likely to be limited in most cases and 2) daily addition of fresh nutrients allows overgrowth by facultative cocci and development of an atypical root canal biofilm, lacking the anaerobic spiral bacterial morphotypes.
After two days and two weeks of anaerobic incubation in BHI broth, twelve CHA biofilms (three biofilms from each of two donors) were prepared for transmission electron microscopy. The samples for thin-sectioning were prepared as described by Lounatmaa (1985). Briefly, the samples were prefixed in phosphate-buffered (pH 7.2) 2.5% glutaraldehyde for 2 h at room temperature and overnight at 4°C. The fixed cells were collected by centrifugation and washed three times with phosphate buffer. All samples were postfixed with phosphate-buffered 1% osmium tetroxide and stained with 4% tannic acid and 4% ruthenium red and dehydrated. The electron micrographs were taken with a JEOL 1200-EX transmission electron microscope at 60 kV.

At 1, 2, 3, 4 and 8 weeks of biofilm growth, specimens from all six donors were rinsed twice in 1 ml of physiological saline for 1 min and immersed in 1 ml of either 1% of sodium hypochlorite, 0.2/0.4% iodine potassium iodide (IPI) or 2% CHX for 1 and 3 min. 1% NaOCl was freshly prepared by diluting a 6% stock solution (RW Packaging, Winnipeg, MB, Canada) in distilled water, 0.2/0.4% IPI was prepared by mixing 0.2 g of iodine (I₂; Sigma Chemical Co, St Louis, MO, USA) in 0.4 g of potassium iodide (PI; Sigma Chemical Co) and adding distilled water to a 100 ml volume, and 2% CHX was freshly prepared by diluting in sterile water from 20% stock solution of chlorhexidine digluconate (Sigma Chemical Co). Each disinfecting solution at each experimental time and growth stage was considered an experimental group. Control specimens, after rinsing in saline, were exposed to 1 ml of sterile commercialized water (Danone Waters of Canada Inc., Richmond, BC, Canada) for 3 min.
After the exposure, biofilms were rinsed in saline for 1 min, stained using LIVE/DEAD BacLight Bacterial Viability kit L-7012 for microscopy and quantitative assays (Molecular Probes, Eugene, OR, USA) and evaluated using confocal laser scanning microscopy (CLSM). The stain contains separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) in solution and was prepared following the manufacturer’s instructions. The excitation/emission maxima for these dyes are between 480/500 nm for the SYTO 9 stain and 490/635 nm for propidium iodide. The mounted specimens were observed using a 10 x lens. Five random areas of the biofilm on each disc were scanned resulting in ten scanned areas for each group. Fluorescence from stained cell was viewed using a CLSM (Nikon Eclipse C1, Nikon Canada, Mississauga, ON, Canada). Simultaneous dual channel imaging was used to display green and red fluorescence. CLSM images of the biofilms were acquired by the EZ-C1 v. 3.40 build 691 software (Nikon) at a field resolution of 512 x 512 pixels and a pixel resolution of 2.5 μm giving the scanned field area of 1.64 mm². Typically, confocal images of ≈120 slices were captured from top to the bottom of the biofilm (each slice was 0.5 μm thick). Obtained LIVE/DEAD images were analyzed and quantified using the bioImage_L software (http://www.bioimageL.com/get_bioimage_L for 2-D analysis) (Chávez de Paz et al. 2009). 3-D analysis was done by extended version of bioImage_L kindly provided by Dr. Luis E. Chávez de Paz. The volume ratio of red fluorescence (dead bacteria) to green-and-red fluorescence (live and dead bacteria) indicated the portion of dead cells in the biofilms at each time. Statistical analysis of the data was done using one-way analysis of variance (ANOVA; SPSS version 11.5 Chicago, IL, USA) and post hoc Tukey test. The significance level was set at p<0.05.
2.3  Results

2.3.1  TEM Examination of the *In vitro* Biofilm

Figures 2-10 are representative from what was seen after scanning twelve biofilms (from two donors) using TEM. A variety of bacterial morphotypes were present in this *in vitro* biofilm model (Figure 2 and Figure 3) mostly consisting of cocci and rods of different sizes and shapes. Gram positive cells were observed in high numbers (Figure 4). Corncob-like formations were also detected throughout the biofilm. In cross-sections, about 8-10 cocci could be found surrounding the central filament (Figure 5). The cocci ranged in diameter from 0.4 to 0.8 µm, but frequently they assumed more oblong shape (Figures 2-10). The rods consisted of cells approximately 0.6-1 µm diameter and 3-5 µm in length. The long rod cells present in biofilm could potentially represent species *Rotia dentocariosa*, *Leptotrichia buccalis* or *Actinomyces* spp. (Figure 6). The cell wall structure of most of cell types was compatible with that described for Gram-positive bacteria (Glauert & Thorney 1969). The overall thickness of the coccal wall varied from approximately 20-40 nm (Figure 4). Prominent surface structures, possibly proteins or glycoproteins, were seen on many bacteria (Figure 4). Some bacteria had damaged cell membranes (Figure 7). Many bacteria were in the process of division (Figures 8, 9). Endospores could also be observed throughout the biofilm (Figure 10). The extracellular polymeric substance was not observed.
Figure 2. TEM micrograph of a thin sectioned specimen of different types of bacteria in the *in vitro* biofilm (7,500 X magnification). Tannic acid and ruthenium red staining in this and subsequent images
Figure 3. Thin sectioned specimen of various bacterial shapes in the biofilm model (25,000 X magnification)
Figure 4. TEM micrograph of bacterial cells with prominent surface structures (arrows) of gram positive bacteria (50,000 X magnification)
Figure 5. TEM image of corncob-like formations (arrows) in the *in vitro* biofilm (10,000 X magnification)
Figure 6. TEM micrograph of different bacterial morphotypes (arrow- a long rod cell; 7,500 X magnification)
Figure 7. Thin sectioned specimen of dead cells with damaged cell membrane (arrow)

(50,000 X magnification)
Figure 8. TEM micrograph of biofilm bacteria showing bacterial cell division (early stage; arrow) (25,000 X magnification)
Figure 9. TEM micrograph of bacterial cell division (late stage; arrow) (50,000 X magnification)
Figure 10. Thin section specimen of endospores (arrows) in the biofilm (10,000 X magnification)
2.3.2 Killing of Bacteria by the Disinfecting Agents

A total of 420 HA biofilm discs and 2,100 scanned areas were analysed for the percentage of viable and killed bacteria. The mixed bacterial biofilms were grown from plaque bacteria sampled from six different donors. Bacteria in one and two week old biofilms were moderately or very sensitive to the disinfecting agents and 20-100% of the bacteria were killed depending on the disinfecting agent and the time of exposure (Figure 10, 11). One percent solution of NaOCl was the most effective disinfectant; it killed almost 100% of the bacteria after 3 min of exposure. At and after three weeks the bacteria in all six biofilms were much more resistant than the bacteria in the young biofilms. The percentage of bacteria killed at and after three weeks of biofilm growth by the same agents using the same exposure times was significantly lower with only 10-30% of the bacteria being killed (p<0.001). After three weeks the proportion of killed bacteria remained relatively unchanged. The same pattern of biofilm resistance development between two and three weeks of biofilm maturation was observed in all six biofilms and with all three disinfecting agents tested.

There was a significant difference in the percentage of killed bacteria by all tested irrigants in young (one and two weeks old) and matured (three weeks and older) biofilms (p<0.001). Also, a significant difference was found between the percentage of eliminated bacteria by 1% NaOCl and 0.2/0.4% IPI and 2% CHX at all tested times in young and matured biofilms (p<0.001). However, a significant difference in the percentage of killed bacteria between 0.2/0.4% IPI and CHX was found in one week old biofilms only (p<0.001).
Figure 11. The percentage of dead bacteria in six different biofilms of different ages after treatment with 1% NaOCl, 0.2/0.4% IPI and 2% CHX for 1 and 3 min. The results shown as the mean ± S.D.
Figure 12. Three-dimensional constructions of CLSM scans of 1-week-old biofilms after exposure to disinfecting agents and viability staining. (A) Biofilm exposed to sterile water (control), (B) 2% CHX exposure for 1 minute, (C) 2% CHX exposure for 3 minutes, (D) 0.2/0.4% IPI exposure for 1 minute, (E) 0.2/0.4% IPI exposure for 3
minutes, (F) 1% NaOCl exposure for 1 minute, and (G) 1% NaOCl exposure for 3 minutes. Green (viable bacteria) and red (dead bacteria)
2.4 Discussion

The effectiveness of different antimicrobial strategies including root canal preparation, the use of irrigating solutions, intracanal dressing, and root filling have been evaluated with somewhat controversial results (Wilson 1996, Siqueira et al. 1998, Abdullah et al. 2005, Clegg et al. 2006, Giardino et al. 2007, Estrela et al. 2009b). Due to the recognised limitations of results from studies of bacteria in planktonic culture, a focus has shifted to the study of biofilms to evaluate the antibacterial effect of endodontic irrigants and other disinfecting agents. While a wide variety of different biofilm models have been used, the focus has been mostly on the antibacterial effects of the disinfecting agents whereas the model itself, the biofilm, has not been well characterized (Duggan & Sedgley 2007, Kishen et al. 2006, Estrela et al. 2009b). Since there has not been a consensus for what constitutes a standardized biofilm model, it has been difficult to compare results from different studies and interpolate them to clinical situations. Therefore, the present study had the following goals: to characterize a recently introduced in vitro multispecies biofilm model grown on CHA discs, and evaluate the effect of the source of biofilm bacteria, the level of biofilm maturation, and the type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents.

In vitro studies on the antibacterial efficacy of endodontic irrigants and medicaments have used biofilm bacteria grown on microscope slides (Williamson et al. 2009), porcelain (Dunavant et al. 2006), polystyrene microtiter plates (Duggan & Sedgley 2007), nitrocellulose membrane (Chai et al. 2007), dentin (Brändle et al. 2008) and hydroxyapatite discs (Shen et al. 2009, Deng et al. 2009). In the present study, HA discs with collagen

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coating and nutrient-rich media were used. Biofilms were grown in conditions that simulate the situation in the root canal [minimal fluid flow and sparse nutrition (fresh medium added only once a week)]. CHA discs chemically resemble dentin (HA and collagen); however, they do not represent the microanatomy of dentin. Dentin is a composite material made up of inorganic (around 70 wt%) and organic fractions (around 20 wt%, mainly collagen). The inorganic portion of dentin is composed primarily of a poorly crystalline-carbonated HA with needle and/or plate-like morphology, existing both within the collagen fibrils (intrafibrillarly mineralized) and between the fibers (interfibrillarly mineralized) (Haapasalo et al. 2007). Type I collagen is the major organic component (90%) of dentin, whereas noncollagenous proteins are present in small amounts. Also, collagen type I is the main ingredient of predentin, the most superficial layer of dentin on the root canal wall which is not yet mineralized (Butler 1984). It is known that certain bacteria through surface adhesins can attach to type I collagen in dentin (Love & Jenkinson 2002) and form biofilms (Jagnow & Clegg 2003, Black et al. 2004). Collagen coating supports bacterial adherence to HA discs; thicker biofilm was formed on collagen-coated hydroxyapatite discs than on HA discs (Shen et al. 2009).

Biofilms grown on polycarbonate or glass substrate may not provide a true indication of the bacteria-substrate interaction. In the present study, HA discs with coating of type I collagen provided a chemically suitable substrate for multispecies biofilm growth. In addition, the standard shape of the discs provided consistent growth of biofilms which was not always possible when other substrates were used. The chemical similarity of the substrate with the teeth/dentin and the steady growth of the multispecies biofilm indicate that this model has potential to be used as a standard biofilm model for in vitro studies of endodontic biofilms.
In the present study, more than 90% of the bacteria were viable for up to eight weeks of culture, including oral spirochetes (spiral morphotypes as observed by SEM), which has not been described before in other multispecies *in vitro* biofilms.

The examination of the new *in vitro* multispecies biofilm model obtained by transmission electron microscopy, complements previously published data obtained by Shen et al. (2009) using scanning electron microscopy. As there is little or no data characterizing biofilm models, one example of a true *in vivo* biofilm (dental plaque) was used as a guide to characterise the bacterial presence in our biofilm model. Listgarten et al. (1973) reported structural observations of bacterial plaque collected on epoxy resin crowns inserted over the mandibular first molar of an adult volunteer for periods of 1, 3, 7 and 21 days. The most prominent findings were bacterial formations resembling ears of corn or corncobs as previously described by Jones (1972). They were noted primarily on the proximal surfaces of the crowns, from the 3-day and older samples. Similar corncob structures were also detected in our biofilm model (Figure 5).

The results of the current study demonstrated that 1% sodium hypochlorite, 0.2/0.4% iodine potassium iodide and 2% chlorhexidine were effective in killing one and two week old biofilm bacteria while bacteria in three week and older biofilms showed increased resistance to these agents. One percent NaOCl was more effective than 0.2/0.4% IPI and 2% CHX against young and mature biofilms. The differences in the percentage of killed bacteria by the tested agents were greater in young biofilms; in older biofilms these differences were less notable. Biofilms used in most of the studies that evaluated the killing effectiveness of endodontic irrigants have been up to seven days old (Clegg et al. 2006,
Dunavant et al. 2006, Williamson et al. 2009). The literature reporting the impact of biofilm age on the efficacy of endodontic disinfecting agents is scarce and so far only one study analyzed the susceptibility of bacteria in biofilms of different ages to CHX products (Shen et al. 2011). The results from this study demonstrated that if young, non-matured biofilms are used to assess the antibacterial efficacy of disinfecting agents, the results would be too optimistic with regard to their effect against bacteria in mature, older biofilms. Most biofilms causing endodontic and periodontal infections are likely to be several weeks, months or even years old at the time of the treatment. It is therefore important to use mature biofilms when evaluating the antibacterial efficacy of endodontic irrigants.

The same pattern of biofilm resistance of three weeks and older was found in all biofilms grown from bacteria of six different donors in the present study. Bacteria for multispecies biofilms were obtained from supra and subgingival plaque of six donors with healthy gingiva, mild gingivitis or periodontitis. Normal oral flora is known to be subject specific (Aas et al. 2005), therefore it is likely that each of the biofilms comprised different bacterial species. The results of the current study strongly indicate that biofilm resistance to different disinfecting agents was more dependent on the maturation process and less dependent on the type of bacteria present in the biofilm or the type of disinfectant used. In our model, the maturation was finished between two and three weeks of biofilm growth.

The central principle of biofilm formation is that it is dynamic. It is often described as a linear process that commences when free-floating bacterial cells attach to a surface. This attachment is followed by growth into a mature, structurally complex biofilm that
culminates in the detachment of bacterial cells into the surrounding fluid (Costerton & Stewart 2001). Growth periods of different lengths are required for biofilms developing in different ecological conditions to reach structural maturity on the basis of microscopically measured physical dimensions and visual comparison. Even though it has been accepted that biofilms require some time to become mature, most of the studies that have evaluated the antibacterial efficacy of endodontic irrigants have used biofilms only at one time point of growth without determining the level of maturation. Therefore, the discrepancy between the proportions of killed bacteria in studies on *in vitro* biofilms (up to 7 days old) and mixed bacterial biofilms in root canals is not surprising. The excellent antibacterial effectiveness of endodontic irrigants reported in the studies on young *in vitro* biofilms has not been confirmed in *in vivo* studies (Paquette et al. 2007, Malkhassian et al. 2009).

Bacteria in mature biofilms are more resistant to disinfectants than planktonic bacteria because of the physical barrier of the biofilm matrix (Hall-Stoodley et al. 2004), physiological state of biofilm bacteria (starved phase) (Hall-Stoodley et al. 2004) as well as the existence of subpopulations known as “persisters” (Suci & Tayler 2003). The results of the present study with six different biofilms and three different disinfecting agents indicate that biofilm resistance is a direct reflection of maturation. However, it is important to point out that although biofilms in the current study reached maturation between two and three weeks of growth, this is not necessarily the case for other biofilm models. Each biofilm model should be analyzed with respect to the length of time required for maturation and decline in bacterial sensitivity to antibacterial measures.

In the current study, all biofilms changed from sensitive to resistant between two and three weeks. While bacteria in one and two-week old biofilms were moderately or very sensitive
to the tested agents, a significant decrease in sensitivity occurred at three weeks. Sauer et al. (2002) characterised five different stages of the development of *P. aeruginosa* biofilms, with the following timeline: >0 min - reversible attachment; 2 hours - irreversible attachment; 3 days - maturation 1, 3-9 days - maturation 2; and 9-12 days - dispersion. They analyzed structural differences and gene expression, and performed protein characterisation in each of these stages. The study suggested that at least nine days were needed for single species biofilm to reach maturation whereas in the current study, *in vitro* biofilms reached maturation between two and three weeks. There are several possible reasons that can explain the observed difference in the time needed for maturation between these two biofilms. Firstly, in the study of Sauer et al. (2002), a single species biofilm was grown while in the current study mixed bacterial biofilms were utilized. Also, Sauer et al. grew biofilms under continuous medium flow, whereas the biofilms in the current study were grown under minimal fluid flow and a limited supply of fresh nutrients. Finally, in their study the level of maturation was determined by protein characterisation while in the current study increased resistance to disinfecting agents suggested that the biofilm has reached maturation. Bearing in mind that the majority of studies have used young biofilms of up to 7 days old, it is possible that only a few if any of those biofilms had reached maturation at the time when they were challenged by the disinfecting agents.

Sodium hypochlorite, chlorhexidine and iodine potassium iodide were chosen for the present study because they are common endodontic irrigants/disinfecting solutions and have different mechanisms of antimicrobial effect. Antimicrobial activity of sodium hypochlorite includes action on bacterial essential enzymatic sites promoting irreversible inactivation. Also, the high pH of NaOCl interferes with bacterial cell membrane integrity.
Furthermore, NaOCl causes cellular metabolism alteration and phospholipid degradation (Mohammadi 2008). It has also been suggested that sodium hypochlorite attacks the microbes' heat shock proteins causing the bacteria to form into clumps and eventually to die (Winter et al. 2008). CHX reacts with bacterial cell membrane (Athanassiadis et al. 2007) and causes the precipitation of the cytoplasmic contents (Gomes et al. 2001) whereas the mechanism of IPI involves multiple cellular effects by binding to proteins, nucleotides and fatty acids (Gottardi 1983). The results from the current study showed that all three disinfecting agents were moderately or very effective in killing bacteria in young biofilms (one and two week old). Interestingly, biofilm bacteria became resistant to all of these agents at the same time, between two and three weeks.

The purpose of the current study was not to rank the tested irrigants but to understand the relationship between antibacterial agents and biofilm development. Therefore, the concentrations of the tested agents were selected to be suitable for the experimental setup of the current study, not to reflect the concentrations recommended for the clinical use. For example, concentrations higher than 1% of sodium hypochlorite make CLSM scanning difficult or impossible due to the bubbles created in the biofilms. Also, 2% and higher NaOCl concentrations often cause detachment and dissolution of young biofilms from the hydroxyapatite discs. Similarly, IPI was ten times diluted from the clinically recommended concentration because at concentration 2/4%, it stains the HA discs and biofilms and dark and fluorescent stains cannot be reliably observed with CLSM. However, recent studies using another new biofilm model with *E. faecalis* in dentin canals, which also allowed high concentrations of NaOCl and other disinfecting agents to be used, indicated a similar time-
dependent development of resistance by the biofilm bacteria (Ma et al. 2011, Wang et al. 2012).

2.4.1 Limitations of the Study

Reliable determination of viability of bacteria is important when evaluating the antimicrobial efficacy of disinfecting agents. Plate count method has been traditionally used for determination of viability of bacteria. However, there are obvious disadvantages of this method with biofilm microorganisms. First, plate count method requires long incubation times (Lahtinen et al. 2006). In addition, the choice of growing medium and incubation conditions that support all different types of bacteria can be a difficult task (Lahtinen et al. 2006). Furthermore, this method is often faced with technical difficulties such as clumping and inhibition of neighboring cells (Lahtinen et al. 2006). Another important problem in bacterial culturing is the presence of “dormant” bacteria in biofilms that are unable to grow on laboratory media (Kell et al. 1998, Shen et al. 2010a).

Due to the obvious limitations of the cultural enumeration techniques, other viability indicators that can assess bacterial viability without culturing cells have been introduced. These indicators are based mostly on fluorescent molecules, which can be detected with epifluorescence microscopy, solid state cytometry, or flow cytometry (Lahtinen et al. 2006). The commercially available LIVE/DEAD BacLight kit (Invitrogen) has enjoyed increasing popularity since it was released about 10 years ago (Boulos et al. 1999). The kit consists of two stains, SYTO 9 and propidium iodide (PI) which both stain nucleic acids. Green fluorescing SYTO 9 is able to enter all cells and is used for assessing total cell
counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. Although viability staining enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells (Gasol et al. 1999, Sachidanandham et al. 2005). While it seems accurate enough to assume that the great majority of membrane-compromised bacterial cells are dead (Berney et al. 2006, Nebe-von-Caron et al. 2000), the reverse (that intact cells are active cells) is not necessarily true (Joux & Lebaron 2000).

Supra- and subgingival plaque bacteria were collected and grown into biofilms under specific conditions in the present studies. Efforts were made to simulate many of the key characteristics of a necrotic root canal environment, where endodontic biofilms grow. These included collagen coated hydroxyapatite, chemically close to dentin, and carbohydrate poor and peptide rich culture medium, which is supposed to reflect the type of nutrients available in a closed, necrotic root canal which is not directly connected to the oral cavity. In addition, fresh nutrients were added only once a week or once a month because of the assumption that in many necrotic root canals the access of microorganisms to nutrients is much more limited than e.g. in a gingival pocket. The fact that e.g. spiral bacterial forms were commonly seen in our new biofilm model may suggest that the biofilm ecology supported fastidious and demanding species that have been described also in true endodontic biofilms. However, despite these efforts and observations, the true similarity of the new model and real in vivo biofilms in necrotic root canals remains to be verified. Even though the composition of the two biofilms (supra and subgingival dental plaque and endodontic biofilm) might be significantly different in terms of the organisms present (the differences being the probable increased number of species in gingival derived
biofilms) many of the endodontic biofilm organisms are found in gingival plaque
(Sundqvist & Figdor 2003). In addition, the various gingival biofilms tested in the current
study were heterogeneous in terms of their development and the resultant climax
communities present (amongst themselves), yet the treatment regimes demonstrated similar
killing curves. It is quite possible that the eradication of endodontic biofilms would follow
similar killing dynamics. However, definitive proof would require development of a
number of biofilms from root canals.

2.5 Conclusion

In conclusion, the results of the current study suggest that Shen et al. (2009) in vitro biofilm
model shares some key features with in vivo oral biofilms as described in the literature and
therefore regarded of being suitable as a model for endodontic research. Also, the results
showed that bacteria in mature biofilms were more resistant to 1% sodium hypochlorite,
0.2/0.4% iodine potassium iodide and 2% chlorhexidine than bacteria in young biofilms. A
change in bacterial sensitivity occurred between two and three weeks in all biofilms
irrespectively of the source of the biofilm bacteria or the type of disinfectant used. This
change is probably a consequence of biofilm maturation. After three weeks no changes in
bacterial sensitivity were detected and the killing ratios remained constant throughout the
rest of the eight week test period. Knowing the maturation timeline of each biofilm model
is important when evaluating the effectiveness of endodontic disinfecting agents against
biofilm bacteria.
Chapter 3: *Ex vivo* Killing of *Enterococcus faecalis* and Mixed Plaque Bacteria in Planktonic and Biofilm Culture by Modified Photoactivated Disinfection

\(^2\) A version of this chapter was published. Stojicic S, Amorim H, Shen Y, Haapasalo M. *Ex vivo* killing of *Enterococcus faecalis* and mixed plaque bacteria in planktonic and biofilm culture by modified photoactivated disinfection. Int Endod J 2013;46:649-59.
3.1 **Introduction**

The goal of endodontic treatment is to obtain a clean root canal, free of bacteria before obturation in order to allow periapical healing. It has been shown that bacteria remaining after completed endodontic therapy may lead to persistent or recurrent infection (Byström et al. 1987, Haapasalo et al. 2008). Chemomechanical preparation of the root canal by instrumentation and irrigation often fails to eliminate all bacteria. This is partly due to anatomical irregularities sometimes inaccessible to endodontic instruments or disinfectant delivery (Nair et al. 2005). Furthermore, bacteria that persist in biofilms show a wide range of characteristics that differ from planktonic cells, including increased resistance to antimicrobial agents. Biofilms also protect microorganisms from host defense. They facilitate nutrient uptake, removal of metabolic products and development of appropriate physicochemical environment (Estrela et al. 2009b). On the other hand, disinfectants able to kill biofilm bacteria can be harmful for periapical tissue (Gernhardt et al. 2004, Barnhart et al. 2005) or may have a negative impact on mechanical properties of dentin (Ari et al. 2004, Mareanding et al. 2007).

Photoactivated disinfection has been introduced to dentistry as a host friendly way of attacking microorganisms in periodontal and endodontic infections. While most other substances or methods used in root canal disinfection are directly (hypochlorite) and potentially (antibiotics) harmful to the host, PAD is claimed to target microorganisms specifically with no collateral damage to host tissues. It involves the use of a photosensitizer (usually dye) that is activated by light in the presence of oxygen. During the first phase, a photosensitizer binds to bacterial membrane and enters into cytoplasm. When
exposed to the light of a specific wavelength, a photosensitizer becomes excited and reaches higher levels of energy state. It reacts with either oxygen or other biomolecules and creates singlet oxygen and free radicals. Both of these products are highly reactive and lead to damage of vital cell functions and cell death (Konopka & Goslinski 2007).

The history of PAD begins in early 20th century when Paul Ehrlich demonstrated that bacteria can selectively accumulate vital stains, such as methylene blue (Wainwright 1998). Even though the interest for its antibacterial properties was hampered by the introduction of antibiotics (1930’s), PAD research has been revitalized in recent years partly because of the continuously increasing challenges with antibiotics. PAD represents a potential alternative methodology to inactivate microbial cells (Taylor et al. 2002) and has already shown to be effective as an in vitro antibacterial procedure (Wainwright 1998, Smith 2005, Soukos et al. 2006, Bergmans et al. 2008). However, while many studies initially demonstrated promising results using PAD against oral bacteria, several recent studies have shown that the effect of PAD in fact seems to be inferior to many of the more established methods, such as use of sodium hypochlorite in disinfection of the dental root canal (Soukos et al. 2006, Bergmans et al. 2008). However, one of the assumed advantages of PAD, safety, remains a valid goal in disinfection.

George & Kishen (2007) demonstrated that bacterial killing can be improved by modifying photosensitization and irradiation medium and by introducing dual stage approach in PAD [stage I- exposure to a photosensitizer (preincubation time, PIT), stage II- laser irradiation]. Also, an advanced formulation which contained oxidizer and oxygen carrier improved the
effectiveness against biofilm bacteria considerably (George & Kishen 2008). Nevertheless, neither conventional nor improved PAD protocols were able to kill all bacteria.

The aim of this study was to compare the efficacy of a commonly used (“conventional”) and a new, modified photoactivated disinfection protocol in the killing of *E. faecalis* and mixed plaque bacteria in suspension and biofilms. The null hypothesis was that there is no difference between conventional and modified PAD in the *ex vivo* killing of *E. faecalis* and mixed plaque bacteria in either planktonic or biofilm culture.

### 3.2 Materials and Methods

#### 3.2.1 Microorganisms

##### 3.2.1.1 *E. faecalis* Suspension

Four strains of *E. faecalis* (VP3-181, VP3-180, Gel 31 and Gel 32) originally isolated from root canals of the teeth with periapical lesions (Peciuliene et al. 2000) were grown overnight on Tryptic Soy Agar (TSA) plates (Becton Dickinson) at 37°C. Bacteria were harvested from the plates using an inoculum loop, checked for purity and collected in sterile water (Danone Waters of Canada Inc.). The cells in suspension were adjusted for optical density (OD) 0.25, measured in 150 μl at 405 nm by a microplate reader (Model 3350, Bio-Rad Laboratories, Richmond, CA, USA), corresponding to $2.25 \times 10^8$ CFU/ml as determined by serial tenfold dilutions in BHI broth (Becton Dickinson) and aerobic culturing on TSA plates for colony forming units (CFU) counts.
3.2.1.2 Mixed Plaque Suspension

Plaque was collected from three healthy volunteers with a wooden stick from several supra and subgingival sites in the molar and premolar region. The plaque was suspended in sterile water as described above and adjusted to OD = 0.1 which corresponded to $6 \times 10^6$ CFU/ml.

3.2.1.3 Biofilms

Sterile hydroxyapatite discs (9.6 mm diameter by 1.5 mm thickness; Clarkson Chromatography Products) were coated with bovine dermal type I collagen (10 μg/ml collagen in 0.012 N HCl in water; Cohesion) and used as biofilm substrate (Shen et al. 2009, 2010a, 2010b). *E. faecalis* (VP3-181) grown overnight on TSA plates or subgingival plaque was suspended in BHI broth. Coated HA discs were placed in the wells of a 24-well tissue culture plate (Costar) containing 1.8 ml of BHI broth and 0.2 ml of either *E. faecalis* or plaque suspension was added to each well. The discs were incubated under anaerobic conditions (AnaeroGen, OXOID) at 37°C for 21 days. Culture medium was changed once a week.

3.2.2 Solutions and Laser Irradiation

Methylene blue (MB) stock solution was made by adding 0.096 g of MB powder (Sigma Chemical Co.) into 100 ml of distilled water giving a concentration 3,000 μM (960 μg/ml). The different MB solutions (300, 200, 100, 60, 30 and 15 μM) were prepared by diluting the stock solution in distilled water. For modified PAD the following additional solutions
were made: CHX in concentration of 0.1% was prepared by diluting the 20% stock solution (Sigma Chemical Co.) in distilled water, 0.1% EDTA was made by diluting the 50% stock solution (Sigma Lab Chem. Inc, Pittsburgh, PA, USA) in distilled water, and 0.1% and 1% hydrogen peroxide (H$_2$O$_2$) were prepared from the 30% stock solution (Fisher Scientific, Ottawa, ON, Canada) by diluting in distilled water just before use. To compare the efficacy of PAD in biofilm killing to conventional disinfection, 1% and 2% NaOCl prepared by dilution of 6% stock solution (RW Packaging) in distilled water and 2% CHX obtained by diluting 20% stock solution (Sigma Chemical Co.) in distilled water were used.

In order to determine the optimal photosensitizer (MB) concentration for killing planktonic bacteria, *E. faecalis* (VP3-181) suspension (0.5 ml) was incubated with MB solutions (0.5 ml) in concentrations 300, 200, 100, 60, 30 and 15 μM (giving the final concentration of MB 150, 100, 50, 30, 15 and 7.5 μM) for 5 min and then exposed to laser for 30 s, 1 and 3 min. Samples were taken from the suspension and CFU was counted after serial tenfold dilutions in BHI broth and culturing on TSA plates. Based on these experiments (Table 1), 15 μM (final concentration) was determined as the most effective concentration and therefore used for killing bacteria in planktonic phase by conventional and modified photoactivated disinfection.

Similarly, the most effective concentration of MB against biofilm bacteria was determined. After 5 min preincubation time in 1 ml of MB solutions 150, 100, 50, 30 and 15 μM (final concentrations) biofilms were irradiated by laser for 1 min. Using LIVE/DEAD BacLight Bacterial Viability kit L-7012 (Molecular Probes) and confocal laser scanning microscope (Nikon Eclipse C1, Nikon Canada) the percentage of killed bacteria was calculated.
(detailed procedure described on pages 77 and 78). A concentration of 100 $\mu$M of MB was the most efficient and it was used in killing biofilm bacteria by conventional and modified PAD (Table 6).

The irradiation source was Twin Laser (Diode Laser, MM Optics, Sao Paolo, Brazil) with an output power of 40 mW and wavelength of 660 nm. Two different tips were used for light delivery. A long optical fiber with a diameter 0.4 mm was used for the irradiation of suspensions, while a conical frustum tip with the end diameter of 5 mm was used to deliver light to the biofilm.

3.2.3 Conventional Photoactivated Disinfection

3.2.3.1 Planktonic Killing

Suspensions (0.5 ml) of four strains of $E. faecalis$ were exposed to laser irradiation for 30 s, 1 and 3 min, after being preincubated with 30 $\mu$M MB (0.5 ml) (final concentration 15 $\mu$M) for 0 or 5 min. Laser treatment was done in 1.8 ml Eppendorf test tubes. Immediately after irradiation, bacterial samples were taken, serially diluted in BHI broth and cultured on TSA plates. The number of colonies was counted after 24 and 48 hours of incubation at 37°C in air.

Suspensions of mixed plaque bacteria (0.25 ml) were exposed to laser light for 1, 3 or 5 min with or without preceding incubation (PIT= 1, 3 or 5 min) with 0.25 ml of 30 $\mu$M MB solution (final concentration 15 $\mu$M). Samples were taken at indicated times and cultured
on blood agar plates [BHI Agar (Difco, Detroit, MI, USA) with 5% heparinized sheep's blood] after serial tenfold dilutions in BHI broth. The growth was measured after 72 h of anaerobic incubation (AnaeroGen, OXOID) and followed for up to two weeks for possible delayed growth.

The following controls were used for the experiments: 1) *E. faecalis* (all strains) or mixed plaque bacteria incubated with commercialized sterilized water (Danone Waters of Canada Inc.) with no MB or laser irradiation, 2) *E. faecalis* (all strains) or mixed plaque bacteria incubated with MB but no laser irradiation and 3) *E. faecalis* (all strains) or mixed plaque bacteria incubated in sterilized water exposed to laser.

3.2.3.2 Biofilms

After 21 days of biofilm growth, CHA discs were rinsed twice in 1 ml of physiological saline for 1 min to remove the culture broth. The discs were immersed in 1 ml of MB (100 μM) and exposed to laser irradiation for 1 min with PIT of 0 and 5 min. Laser was applied on the top of the discs for 1 min. During laser irradiation the discs were kept immersed in MB solutions. Control discs were exposed to sterile water with no MB or laser irradiation, sterile water with applied laser irradiation or MB solutions with no laser irradiation. Three discs were examined for each group; five random areas of the biofilm on each disc were scanned. LIVE/DEAD BacLight Bacterial Viability stain (kit L-7012, Molecular Probes) for microscopy and quantitative assays containing separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) in solution was used for staining of the biofilm. Fluorescence from the stained cells was evaluated using the CLSM. The mounted
specimens were observed using 10 x lenses with a numerical aperture of 0.30. CLSM images of the biofilms were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon) at a pixel resolution of 2.5 μm and a field resolution of 512 x 512 pixels. Each stack had a field area of 1.64 mm². The scans were performed with a 0.5 μm step size. About one hundred scans were acquired of each stack. CLSM LIVE/DEAD images were analyzed and quantified using the bioImage_L software (Chávez de Paz, 2009) (available from http://www.bioimageL.com/get_bioimage_L, for 2-D analysis). 3-D analysis was done using the extended version of bioImage_L kindly provided by Dr. Luis E. Chávez de Paz. The proportion of red fluorescence (dead bacteria) to green-and-red fluorescence (dead and live bacteria) indicated the percentage of dead cells in biofilm. Statistical analysis was done using ANOVA and post hoc Tukey test (SPSS version 11.5).

3.2.4 Modified Photoactivated Disinfection

In modified photoactivated disinfection four different strains of E. faecalis (0.5 ml) or mixed plaque (0.25 ml) suspension were added to corresponding volumes of the mixture of 1) 30 μM MB, 1% H₂O₂, 0.1% CHX, 2) 30 μM MB, 1% H₂O₂, 0.1% EDTA or 3) 30 μM MB, 0.1% of EDTA, 0.1% CHX (giving the following final concentrations: MB 15 μM, 0.5% H₂O₂, 0.05% EDTA, 0.05% CHX) and exposed to laser irradiation for 10, 20, 30 and 60 s. As controls, all solutions (0.5% H₂O₂, 0.05% EDTA and 0.05% CHX) were tested with and without MB and/or laser irradiation to assess if chemical killing without laser played a role in the overall killing of the microbes.
Following the exposure of *E. faecalis*, 10 μl samples were taken, serially diluted in BHI broth and grown aerobically on TSA plates at 37°C for 24 and 48 hours. For mixed plaque bacteria aliquots of 100 μl were serially diluted in BHI broth and grown anaerobically on blood agar plates at 37°C for 72 hours and observed for up to two weeks. The number of viable organisms (CFU) of *E. faecalis* or mixed plaque bacteria was counted for each experiment. The percentage of killed bacteria was determined as the difference in the percentage of living cells after exposure to conventional or modified PAD and the living cells in the initial inoculum.

Three weeks old biofilms (*E. faecalis* and mixed plaque) were immersed in 1 ml of MB (100 μM) for 5 min PIT and then exposed to 1 min laser irradiation after addition of 0.1% EDTA and either 0.1% H₂O₂ or 0.1% CHX. As controls, biofilms were exposed to the mixtures of test solutions with and without addition of MB and/or exposure to the laser irradiation.

In order to compare the efficacy of photoactivated disinfection (conventional and modified) to conventional endodontic irrigants in killing biofilm bacteria, three weeks old biofilms (mixed plaque bacteria and *E. faecalis*) were immersed in 1 ml of 2% CHX, 1% and 2% NaOCl for 1 min. Measurement of bacterial killing in biofilms by modified PAD and conventional endodontic irrigants was carried out as described for conventional PAD.
### 3.3 Results

#### 3.3.1 Planktonic Killing

In order to optimize the bactericidal concentration of MB, *E. faecalis* strain VP3-181 was exposed to different concentrations of MB for three different time periods after PIT of 5 min (Table 1). The final concentration of 15 μM MB was the most effective against planktonic *E. faecalis* at all tested times and was used for the experiments.

To assess the importance of preincubation time, four strains of *E. faecalis* (VP3-181, VP3-180, Gel 31 and Gel 32) were challenged by conventional PAD after PIT of 0 or 5 min (Table 2). After three minutes of laser irradiation, three out of four tested strains were completely killed. Strain Gel 31 was the most resistant of the four tested *E. faecalis* strains and 0.75% of the cells were still alive after 5 min PIT and 3 min of laser irradiation. Killing of the two resistant strains Gel 31 and Gel 32 by conventional PAD was improved after applying the 5 min PIT. Contrary to *E. faecalis*, conventional PAD could not kill all mixed plaque bacteria even after 5 min of laser irradiation. The effect of PIT was minimal on overall bacterial killing (Table 3).

Modified PAD was more effective in killing bacteria than the conventional PAD. The time for complete or almost complete killing of *E. faecalis* by PAD supplemented with 0.05% EDTA, 0.05% CHX and 15 μM MB was reduced from three minutes to one minute (Table 4). When EDTA with either H₂O₂ or CHX was added to MB and incubated with planktonic
plaque bacteria, the time required for complete or nearly complete eradication of bacteria was reduced from 5 min to 30 s (Table 5).

In control experiments, mixtures of chemicals (MB and low concentrations of EDTA, CHX and H₂O₂) were able to kill from 23.91% to 99.73% of planktonic *E. faecalis* and mixed plaque bacteria after 1 minute of exposure. However, none of the chemical mixtures used was able to kill 100% of the planktonic bacteria during the exposure time.

### 3.3.2 Biofilm Killing

The most efficient concentration of MB against *E. faecalis* and mixed plaque biofilms by photoactivated disinfection was 100 μM (Table 6) and about five (*E. faecalis*) and nine (mixed plaque) times more bacteria were killed as compared to conventional PAD using 15 μM MB (the most commonly recommended concentration) (Table 7). Preincubation time played an important role in the killing of biofilm bacteria. Up to almost three times more bacteria were killed by PAD using elevated concentration of MB (100 μM) after 5 min PIT as compared to 0 min PIT (Table 7).

Addition of low concentrations of 0.1% EDTA and 0.1% H₂O₂ or 0.1% CHX to MB 100 μM (modified PAD) improved bacterial killing considerably. Modified PAD was more effective against *E. faecalis* biofilms by killing about 30% more bacteria than PAD using elevated MB concentration (100 μM) and almost eight times more bacteria than conventional PAD (MB 15 μM). Killing of bacteria in mixed bacterial biofilm was enhanced between 30% (mixture of MB, EDTA and H₂O₂) and 175% (mixture of MB,
EDTA and CHX) by modified PAD as compared to PAD using elevated concentration of MB (100 μM) and almost twenty two times more bacteria were killed by modified PAD than by conventional PAD with MB 15 μM (Table 7).

PAD with elevated concentration of MB (100 μM) was about equally effective as 2% NaOCl and more effective than 2% CHX and 1% NaOCl in killing bacteria in plaque bacteria biofilms. About twice as many mixed biofilm bacteria were killed using modified PAD with 100 μM MB, 0.05% EDTA and 0.05% CHX than with 2% NaOCl and up to almost six times more than with 1% NaOCl and 2% CHX. Up to around eight times more E. faecalis in biofilms were killed by modified PAD than by 2% CHX. All traditional endodontic irrigants (2% CHX and 1 and 2% NaOCl) were more effective in killing mixed plaque biofilm bacteria than PAD utilizing 15 μM MB. 2% CHX was almost equally effective as PAD utilizing 15 μM MB against E. faecalis biofilms.
Table 1. Percentage (mean ± S.D.) of killed planktonic *E. faecalis* (VP3-181) by photoactivated disinfection after 5 min preincubation time with different concentrations of methylene blue [MB (μM)]. CFU counted on TSA plates

<table>
<thead>
<tr>
<th>Laser irradiation</th>
<th>MB 150</th>
<th>MB 100</th>
<th>MB 50</th>
<th>MB 30</th>
<th>MB 15</th>
<th>MB 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>0.23±0.33</td>
<td>18.29±7.67</td>
<td>28.68±7.46</td>
<td>64.11±3.4</td>
<td>98.28±0.13</td>
<td>93.89±3.49</td>
</tr>
<tr>
<td>3 min</td>
<td>53.67±15.54</td>
<td>88.82±13.13</td>
<td>99.93±0.01</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2. Percentage (mean ± S.D.) of killed planktonic *E. faecalis* (four strains) by PAD using 15 μM MB, with or without 5 min PIT. CFU counted on TSA plates.

<table>
<thead>
<tr>
<th>E. faecalis</th>
<th>PIT: 0 min</th>
<th>PIT: 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 s Laser</td>
<td>1 min Laser</td>
</tr>
<tr>
<td>VP3-181</td>
<td>64.71±4.61</td>
<td>91.02±1.95</td>
</tr>
<tr>
<td>VP3-180</td>
<td>84.65±0.5</td>
<td>98.31±0.79</td>
</tr>
<tr>
<td>Gel 31</td>
<td>0</td>
<td>1.73±2.44</td>
</tr>
<tr>
<td>Gel 32</td>
<td>0.53±0.74</td>
<td>2.76±3.61</td>
</tr>
</tbody>
</table>

PIT, preincubation time
Table 3. Percentage (mean ± S.D.) of killed mixed plaque bacteria in planktonic state by PAD with 15 μM MB and PIT of 0 – 5 mins. CFU counted on blood agar plates

<table>
<thead>
<tr>
<th></th>
<th>PIT: 0 min</th>
<th>PIT: 1 min</th>
<th>PIT: 3 min</th>
<th>PIT: 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min Laser</td>
<td>3 min Laser</td>
<td>5 min Laser</td>
<td>1 min Laser</td>
</tr>
<tr>
<td>Plaque 1</td>
<td>91.06 ±2.72</td>
<td>99.87 ±0.08</td>
<td>100</td>
<td>93.48 ±2.9</td>
</tr>
<tr>
<td></td>
<td>99.97 ±0.01</td>
<td>99.95 ±0.06</td>
<td>99.99 ±0.02</td>
<td>99.98 ±1.7</td>
</tr>
<tr>
<td>Plaque 2</td>
<td>71.81 ±8.75</td>
<td>99.84 ±0.06</td>
<td>100</td>
<td>87.99 ±8.68</td>
</tr>
<tr>
<td></td>
<td>99.99 ±0.01</td>
<td>99.99 ±0.04</td>
<td>99.99 ±0.02</td>
<td>99.99 ±1.7</td>
</tr>
<tr>
<td>Plaque 3</td>
<td>57.72 ±6.75</td>
<td>99.92 ±0.04</td>
<td>99.99 ±0.01</td>
<td>69.88 ±6.0</td>
</tr>
<tr>
<td></td>
<td>99.99 ±0.01</td>
<td>99.89 ±0.04</td>
<td>100</td>
<td>99.99 ±0.04</td>
</tr>
</tbody>
</table>

PIT, preincubation time
Table 4. Percentage (mean ± S.D.) of killed planktonic *E. faecalis* by combinations of chemical solutions and modified PAD (no preincubation time). CFU counted on TSA plates

<table>
<thead>
<tr>
<th>E. faecalis</th>
<th>A</th>
<th>MB 15 + A</th>
<th>B</th>
<th>MB 15 + B</th>
<th>C</th>
<th>MB 15 + C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>1 min</td>
<td>30 s</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
</tr>
<tr>
<td>VP3-181</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
</tr>
<tr>
<td>31.91</td>
<td>±15.54</td>
<td>41.42</td>
<td>63.56</td>
<td>85.42</td>
<td>39.9</td>
<td>55.6</td>
</tr>
<tr>
<td>VP3-180</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
</tr>
<tr>
<td>57.87</td>
<td>±3.75</td>
<td>36.81</td>
<td>69.11</td>
<td>98.74</td>
<td>47.4</td>
<td>50.85</td>
</tr>
<tr>
<td>Gel 31</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
</tr>
<tr>
<td>40.88</td>
<td>±9.4</td>
<td>23.91</td>
<td>43.74</td>
<td>69.77</td>
<td>25.49</td>
<td>33.63</td>
</tr>
<tr>
<td>Gel 32</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
<td>No laser</td>
<td>No laser</td>
<td>Laser</td>
</tr>
<tr>
<td>39.67</td>
<td>±3.07</td>
<td>63.41</td>
<td>72</td>
<td>94.04</td>
<td>39.69</td>
<td>41.67</td>
</tr>
</tbody>
</table>

A, 0.05% CHX+ 0.5% H2O2  B, 0.05% EDTA+ 0.5% H2O2  C, 0.05% CHX+ 0.05% EDTA
Table 5. Percentage (mean ± S.D.) of killed planktonic plaque bacteria by combinations of chemical solutions and modified PAD (no preincubation time). CFU counted on blood agar plates

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>MB 15 + A</th>
<th>MB 15 + B</th>
<th>MB 15 + C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min laser</td>
<td>30 s laser</td>
<td>1 min laser</td>
<td>1 min</td>
<td>10 s</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No laser</td>
<td>No laser</td>
<td>No laser</td>
</tr>
<tr>
<td>Plaque 1</td>
<td>92.11 ±0.11</td>
<td>91.74 ±1.95</td>
<td>94.68 ±0.59</td>
<td>40.28 ±9.87</td>
<td>53.03 ±0.44</td>
<td>92.80 ±1.6</td>
</tr>
<tr>
<td>Plaque 2</td>
<td>70.56 ±2.55</td>
<td>74.09 ±3.14</td>
<td>65.76 ±7.71</td>
<td>38.75 ±0.5</td>
<td>38.27 ±1.77</td>
<td>78.45 ±9.94</td>
</tr>
<tr>
<td>Plaque 3</td>
<td>N.D. ±2.48</td>
<td>N.D. ±0.25</td>
<td>N.D. ±0.16</td>
<td>N.D. ±1.77</td>
<td>N.D. ±9.94</td>
<td>74.73 ±2.48</td>
</tr>
</tbody>
</table>

A, 0.05% CHX+ 0.5% H₂O₂  B, 0.05% EDTA+ 0.5% H₂O₂  C, 0.05% CHX+ 0.05% EDTA

N.D. not done
Table 6. Percentage (mean ± S.D.) of killed biofilm bacteria exposed to PAD at different concentrations of MB (µM) (1 min of laser irradiation, 5 min preincubation time). Viability staining and CLSM

<table>
<thead>
<tr>
<th>Biofilm</th>
<th>Control*</th>
<th>MB 150</th>
<th>MB 100</th>
<th>MB 50</th>
<th>MB 30</th>
<th>MB 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3-181</td>
<td>1.33±0.58&lt;sup&gt;a**&lt;/sup&gt;</td>
<td>3.67±1.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.67±7.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.33±1.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.33±1.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.67±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plaque</td>
<td>1.6±1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.33±3.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.16±5.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.21±1.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.24±1.45&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Control, biofilms exposed to 1 ml of sterile water for 6 min.

**Different superscript letters suggest significant difference between MB concentrations within plaque biofilms or *E. faecalis* VP3-181 biofilms.
Table 7. Percentage (mean ± S.D.) of killed bacteria in 3-week old biofilm by modified PAD. Viability staining and CLSM

<table>
<thead>
<tr>
<th>Biofilm</th>
<th>VP3-181</th>
<th>Plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>1.33±0.58a</td>
<td>1.60±1.12a</td>
</tr>
<tr>
<td>0.1% EDTA + 0.1% H$_2$O$_2$ 1 min</td>
<td>3.60±2.44a</td>
<td>3.20±2.04a</td>
</tr>
<tr>
<td>0.1% EDTA + 0.1% CHX 1 min</td>
<td>6.33±3.27a</td>
<td>5.70±2.50ab</td>
</tr>
<tr>
<td>MB 15 (PIT=5 min) 1 min LASER</td>
<td>3.67±0.58a</td>
<td>2.24±1.45a</td>
</tr>
<tr>
<td>MB 100 (no PIT) 1 min LASER</td>
<td>7.21±3.67a</td>
<td>6.67±1.15ab</td>
</tr>
<tr>
<td>MB 100 (PIT=5 min) 1 min LASER</td>
<td>20.33±3.51b</td>
<td>18.33±3.51c</td>
</tr>
<tr>
<td>MB 100 (PIT=5 min) + 0.1% EDTA + 0.1% H$_2$O$_2$ 1 min LASER</td>
<td>26.67±1.15c</td>
<td>26.33±0.58d</td>
</tr>
<tr>
<td>MB 100 (PIT=5 min) + 0.1% EDTA + 0.1% CHX 1 min LASER</td>
<td>27.67±0.58c</td>
<td>49.00±5.29c</td>
</tr>
<tr>
<td>2% CHX 1 min</td>
<td>3.40±1.34a</td>
<td>11.90±4.75bc</td>
</tr>
<tr>
<td>1% NaOCl 1 min</td>
<td>N.D.</td>
<td>8.57±4.27ab</td>
</tr>
<tr>
<td>2% NaOCl 1 min</td>
<td>N.D.</td>
<td>22.85±6.82cd</td>
</tr>
</tbody>
</table>

* Control, biofilms exposed to 1 ml of sterile water for 6 min.

**Different superscript letters suggest significant difference between MB concentrations within plaque biofilms or *E. faecalis* VP3-181 biofilms; N.D. not done.
3.4 Discussion

In the current study, *E. faecalis* and mixed plaque bacteria in suspensions as well as in biofilms were challenged by either conventional or modified photoactivated disinfection. One of the acknowledged advantages of PAD is that it is considered safe to host tissues. Moderate side effects such as skin photosensitivity are present only when a photosensitizer is administrated systemically. Systemic photosensitivity does not occur when the photosensitizer is applied topically. Allergic reactions occur infrequently and they are specific for each photosensitizer and each patient (Konopka & Goslinski 2007). Due to the local use of a photosensitizer and laser irradiation, the PAD procedure in endodontics has been considered harmless to patients. On the other hand, the effectiveness of PAD has been shown to be poorer than the effectiveness of many other established methods of disinfection (Lim et al. 2009). It has been suggested that PAD be used as an adjunctive procedure to conventional endodontic treatment (Soukos et al. 2006, Fimple et al. 2008). A number of different photosensitizers in various concentrations have been used in previous studies. Similarly, laser power and time of exposure have varied much (Foschi et al. 2007, Bergmans et al. 2008, Fontana et al. 2009).

In the current study, methylene blue was used as photosensitizer. MB is the most commonly used dye in photoactivated disinfection because of its hydrophilicity, low molecular weight and ability to target both gram-positive and gram-negative bacteria (Harris et al. 2005). The first part of the present study was done in order to assess the importance of photosensitizer concentration for PAD effectiveness. In previous studies, concentration of MB has varied from 6.25 to 100 μg/ml. These studies did not determine
the optimal bactericidal concentration of MB (Soukos et al. 2006, Foschi et al. 2007, Fontana et al. 2009). In the present study, MB in concentrations from 7.5 to 150 μM (2.4 - 48 μg/ml) were tested against planktonic and biofilm bacteria. Better killing of planktonic bacteria was detected using low concentrations of methylene blue, and the concentration of 15 μM MB was shown to be optimal for killing. Enhanced killing of bacteria in suspensions using low concentrations of MB can be explained by its state of existence in different solutions. By measuring the light absorption property of MB, it has been shown that the steady state of MB can vary in different formulations. When dissolved in water, at high concentrations MB exist as dimers rather than monomers (Patil et al. 2002, George & Kishen 2007). Since dimer formation can decrease singlet oxygen production (Gabrielli et al. 2004), low concentrations of MB kill bacteria better when the monomer/dimer ratio is higher. However, a high concentration of methylene blue (100 μM) was optimal for killing biofilm bacteria in the present study. In planktonic culture, MB can easily reach the bacteria in its original concentration, whereas in biofilms, dilution of the penetrating dye is likely to reduce the effective concentration considerably. Therefore, it is not surprising that a high concentration of 100 μM MB was required for best efficacy in biofilm killing. However, concentrations at higher than 100 μM were less efficient than a 100 μM MB solution. In addition to wide variation in dye concentration in previous studies, there are also other factors that make it challenging to compare the results of these studies. In several studies the culture broth (BHI) has been used as the liquid phase for MB in PAD. Since BHI or other media are able to absorb most of the laser energy (Meire et al. 2009), in the current study MB was dissolved in sterile water.
*E. faecalis* was chosen for the experiment because it is one of the most common bacteria in persistent endodontic infections (Molander et al. 1998, Peciuliene et al. 2000, Pinheiro et al. 2003). Also, this gram positive, facultative coccus can be easily cultured. Earlier studies have shown that different strains of *E. faecalis* vary in their susceptibility to disinfectants (Portenier et al. 2005). Therefore, in the current study, four different strains isolated from the root canal of teeth with periapical lesions were used for the experiments. In addition, it is known that the physiological state of bacteria has an impact on bacterial resistance to antibacterial agents. Starved cells survived in numbers 1,000 to 10,000 times higher than the cells in exponential or stationary phase when challenged by different disinfecting agents (Portenier et al. 2005, Liu et al. 2010). In the current study, all tested strains were grown for 24 hours to reach the stationary phase (Portenier et al. 2005).

The results from the current study suggest that the time required for complete killing by conventional PAD of *E. faecalis* and mixed plaque bacteria even in suspension is too long to be practical for clinical use. It should be emphasized that although plaque bacteria in suspension were more susceptible to killing than biofilm bacteria, they were freshly obtained from mature dental plaque immediately before use in the experiments. Therefore, the plaque bacteria were likely to be in a metabolically inactive (starvation) phase and thus much more resistant to disinfection/killing than bacteria in exponential or stationary growth phase. Pooled plaque samples contained supragingival and subgingival microorganisms. As subgingival microorganisms live in an ecologically different environment from supragingival bacteria, it is possible that if only sub- or supragingival microorganisms had been collected, the time required for killing might have been different. However, it has been demonstrated that pooled supra and subgingival plaque provides the most complete
A collection of intraoral pathogens associated with periodontal infections (Beikler et al. 2006). This is also most likely the case for endodontic pathogens.

The effectiveness of conventional PAD was clearly poorer against biofilm bacteria than against bacteria in planktonic state. Biofilms are microcommunities where bacteria exist in slow-growing or starvation phase and can survive harsh conditions (Brown et al. 1988). It has also been shown that biofilm bacteria express distinct and protective phenotypes (Whitley et al. 2001). They can inactivate methylene blue (Foley & Gilbert 1996), or the dye is unable to reach all parts of the biofilm (Stewart 2003).

For optimized PAD, efficient combinations of photosensitizer and light as well as “tissue specific conditions” should be obtained (George & Kishen 2008). The “hypooxygenic” nature of sites infected by bacteria can affect the efficacy of PAD killing because molecular oxygen is a prerequisite for the generation of singlet oxygen. George & Kishen (2008) tested four different photosensitizing formulations containing MB and oxygen carrier alone (perfluoro-decahydronaphthalene) or in combination with oxidizer (H₂O₂) or emulsions formed with nonionic detergent (triton-X100) in different proportions. They found improved PAD more effective against E. faecalis biofilms than conventional PAD. Lim et al. (2009) using a different oxygen carrier showed that improved PAD was superior to chemomechanical disinfection against four-day and four-week old E. faecalis biofilms. While they tried to potentiate the antibacterial efficacy of the oxygen species, the focus of the current study was to attack bacterial cells using different chemical and physical mechanisms to achieve an additive or synergistic effect (oxygen species in PAD and chemical action of various disinfectants in lower concentrations then when used alone).
In the present study, low concentrations of EDTA, H$_2$O$_2$ or CHX in various combinations were added to the MB solution to facilitate bacterial killing. In this modified PAD, the time required to kill *E. faecalis* in suspension was three times shorter than with conventional PAD, and the time to kill mixed plaque bacteria was five times shorter. The two different modifications of PAD (MB concentration and added chemicals) resulted in the improved killing of biofilm bacteria. First, much more bacteria were killed by increasing the concentration of MB from 15 to 100 μM. Further improvement was achieved by adding low concentrations of EDTA, H$_2$O$_2$ or CHX to 100 μM MB. The order of effectiveness of killing biofilm bacteria was as follows: modified PAD (100 μM MB) > 2% NaOCl ≥ PAD (100 μM MB) > 1% NaOCl ≥ 2% CHX ≥ conventional PAD (15 μM MB).

Improved killing of bacteria by PAD after addition of H$_2$O$_2$ to MB has been shown previously (Mc Cullagh & Robertson 2006, Garcez et al. 2011). Garcez et al. (2011) indicated that the addition of hydrogen peroxide to MB during PAD was not related to the higher production of reactive oxygen species in the solution, rather, hydrogen peroxide increased bacterial cell permeability. Also, H$_2$O$_2$ facilitated MB reduction by the cell during photosensitizer intake and oxidation inside the cell, which lead to increased ROS formation inside the cell and eventually improved bacterial killing.

EDTA is a negatively charged molecule reported to cause cell wall damage in gram-negative bacteria by chelating divalent cations (Mg$^{2+}$ and Ca$^{2+}$) (Leive 1974, George et al. 2009). Removing the cations causes the bacterial cell membrane to become more permeable allowing increased penetration of anionic and cationic dyes through the outer membrane (George et al. 2009). It has also been shown that EDTA can remove some
surface components of gram positive *E. faecalis* (Hancock 1984, Smith et al. 1986, George et al. 2009). When used alone, the antibacterial effect of EDTA is at best weak even in high concentrations. However, together with other substances, it can contribute in a synergistic way to weakening of the microbial cell wall.

Chlorhexidine has a long history of use in endodontics for its antimicrobial effect (Delany et al. 1982, ErCAN et al. 2006). Positively charged hydrophobic and lipophilic molecules of chlorhexidine interact with phospholipids and lipopolysaccharides on the cell membrane, destabilize it and increase its permeability. When used at low concentrations (0.2%), CHX will cause small molecular weight substances to leak out of the cell. Higher concentrations (2%) will cause precipitation of the cytoplasmatic contents (Mohammadi & Abbott 2009).

A synergistic effect of CHX and H$_2$O$_2$ against *E. faecalis* has been reported earlier (HelING & Chandler 1998, Steinberg et al. 1999). According to the authors, the synergistic effect was probably gained by the action of CHX on the bacterial wall and H$_2$O$_2$ on the intracellular organelles, such as DNA. However, the exact mechanism is not completely understood (Steinberg et al. 1999). In the present study, this combination of these two solutions was tested in modified PAD, but it was less effective than the other two combinations with EDTA. The mixture of low concentrations CHX and EDTA with MB in modified PAD was the most effective against both planktonic cultures and biofilms of *E. faecalis* and plaque bacteria.
3.5 **Conclusion**

Modified PAD was superior to conventional PAD against *E. faecalis* and mixed plaque bacteria in planktonic phase and biofilms. Optimizing the methylene blue concentration and addition of low concentrations of EDTA with either hydrogen peroxide or chlorhexidine to methylene blue followed by laser irradiation resulted in eradication of planktonic bacteria in a much shorter time and greatly improved killing of biofilm bacteria. Modified PAD was about two to eight times more effective against biofilm bacteria than 2% NaOCl, 1% NaOCl and 2% CHX. The modified PAD may be a viable option for killing biofilm bacteria in infected root canals.
Chapter 4: Antibacterial Efficacy of a Novel Irrigant, QMiX³

³ A version of this chapter was published. Stojicic S, Shen Y, Qian W, Johnson B, Haapasalo M. Antibacterial and smear layer removal ability of a novel irrigant, QMiX. Int Endod J 2012;45:363-71.
4.1 Introduction

Bacteria are the main cause of periapical disease (Kakehashi et al. 1965). The main goal of endodontic treatment is elimination of bacteria from the root canal and prevention of its recontamination after treatment (Byström & Sundqvist, 1981). It has been reported that the success rate of endodontic treatment was higher when teeth were free of bacteria after chemomechanical instrumentation (Byström et al. 1987, Sjögren et al. 1997). While instruments are important in removal of the infected dentine from the main root canal, irrigants play an indispensable role in areas inaccessible to instruments, such as lateral and accessory canals as well as fins and webs throughout the canal (Hasselgren et al. 1988).

One of the most important requirements for an ideal endodontic irrigant is antibacterial activity. Other desirable characteristics are the ability to dissolve organic and/or inorganic tissue, and to have a lubricant as well as a flushing effect. In addition, the irrigant should not be toxic to the surrounding tissues and not weaken the tooth structure (Haapasalo et al. 2010). None of the present irrigants meets all requirements of an ideal endodontic irrigant. Sodium hypochlorite in concentration from 0.5% to 6% is the most commonly used irrigating solution. It has a strong antibacterial (Byström & Sundqvist 1983, Zehnder et al. 2002, Wang et al. 2012b) and tissue dissolving effect (Rosenfeld et al. 1978, Clarkson et al. 2006, Stojicic et al. 2010). However, it is toxic to periapical tissue (Gernhardt et al. 2004) and has been suggested to degrade the micromechanical characteristics of dentin (Ari et al. 2004). Furthermore, it has no effect on the inorganic part of the smear layer (Baumgartner & Mader 1987). EDTA, used usually in concentration of 17%, dissolves the inorganic portion of dentin and smear layer by chelation and is the most frequently used “second”
solution after NaOCl to complete smear layer removal. Recently, Qian et al. (2011) showed that if NaOCl is used again after EDTA or citric acid as the final antibacterial rinse, it causes marked erosion of the root canal dentin wall.

In the past decade, a lot of effort has been put into developing new irrigants and/or establishing new irrigation protocols in order to facilitate the eradication of microbes from the root canal system. Bio Pure MTAD was introduced to endodontics in 2003. A mixture of tetracycline isomer (doxycycline), citric acid and a detergent, MTAD has been shown to be effective in smear layer removal (Torabinejad et al. 2003b). Another study reported that MTAD was less cytotoxic than calcium hydroxide, eugenol, 5.25% NaOCl and EDTA (Zhang et al. 2003). In addition, in vitro experiments indicated that MTAD has a strong antibacterial effect. It was reported to be more effective than NaOCl and EDTA against E. faecalis (Torabinejad et al. 2003a) and mixed bacteria (Shabahang & Torabinejad 2003). However, some of these results have been challenged by other researchers who found the antibacterial effect of MTAD inferior to 6% NaOCl and 2% chlorhexidine (Ruff et al. 2006, Dunavant et al. 2006). Further, Dunavant et al. (2006) reported that 1% NaOCl (99.78%) killed six times more E. faecalis in biofilms than MTAD (16.08%). Although never reported in vivo, Tay et al. (2006) demonstrated red purple staining of root canal dentine when MTAD was used as final rinse after 1.3% of NaOCl and the dentin blocks were exposed to light following irrigation. The reason for the staining was probably oxidation of doxycycline caused by sodium hypochlorite. However, light was required for the appearance of the staining, as it was not observed when teeth were kept in the dark (Tay et al. 2006). Because of the recognized limitations of all endodontic irrigants, the
development of new and better irrigating solutions for endodontics still remains an area of great interest.

QMiX is a novel endodontic irrigant for smear layer removal with added antimicrobial agents. It contains EDTA, CHX and a detergent. QMiX is a clear solution, ready for use with no chair-side mixing. Mixing EDTA and CHX is known to produce white precipitate (Rasimick et al. 2008). In QMiX this is avoided possibly due to its chemical design. It might be that the addition of a detergent to the mixture of EDTA and CHX blocked some of the active sites on their molecules preventing the interaction between them. Another recent concern in endodontic irrigation is a precipitate containing potentially carcinogenic compound that is formed if sodium hypochlorite is mixed with CHX. Despite the CHX content, mixing QMiX with sodium hypochlorite does not produce a precipitate (data not shown) and the solution does not turn brown/orange.

The aim of this study was to assess the antimicrobial efficacy of QMiX, against *E. faecalis* and mixed plaque bacteria in planktonic culture as well as in biofilms.

4.2 Materials and Methods

4.2.1 Medicaments

The medicaments tested for their antibacterial activity were 1) QMiX (Dentsply Tulsa Dental, Tulsa, OK, USA), 2) Bio Pure MTAD (Dentsply Tulsa Dental), 3) 2% CHX freshly prepared from a 20% stock solution (Sigma Chemical Co), 4) 1% NaOCl and 5) 2% NaOCl
freshly prepared by diluting the 6% stock solution (RW Packaging) in distilled water. Sterile commercialized water (Danone Waters of Canada Inc.) was used as control.

4.2.2 Direct Exposure Test

4.2.2.1 Microorganisms and Experimental Conditions

Two different strains of *E. faecalis* (VP3-181, Gel 31) and mixed plaque bacteria collected from three healthy volunteers were used in the study. *E. faecalis* strains originally isolated from infected root canals (Peciuliene et al. 2000) were grown overnight on Tryptic Soy Agar plates (Becton Dickinson) at 37°C in air, checked for purity and collected in sterile water. Bacterial suspension was adjusted to optical density OD=0.25, measured in 150 μl at 405 nm by a microplate reader (Model 3350, Bio-Rad Laboratories) corresponding to 2.25×10^8 CFU/ml as determined by serial tenfold dilutions in BHI broth (Becton Dickinson) and aerobic culturing on TSA plates for CFU counts. Plaque from three volunteers was collected with a wooden stick from several supra- and subgingival sites in the molar and premolar region, suspended in sterile water and adjusted to 2.4×10^7 CFU/ml. Informed consent was obtained from the human subjects (plaque donors). The study was approved by the ethical board of the university.

Fifty microliter samples of *E. faecalis* or mixed plaque suspension were added to 450 μl of the medicament (QMiX, 2% CHX, MTAD and 1% NaOCl) for 5 sec, 30 sec and 3 min. After indicated times of exposure, 10 μl samples of *E. faecalis* or 100 μl of mixed plaque bacteria were added to 990 and 900 μl BHI broth and serially diluted in 10-fold increments
in BHI broth. The first two tubes in the dilution series contained inactivator [3% Tween 80, 0.3% α-lecithin (both from Sigma Chemical Co) for CHX and MTAD and 0.5% sodium-thiosulfate (Fisher Scientific) for NaOCl] to reduce the carry-over effect of the medicaments. Twenty microliters from the dilution tubes were cultured at 37°C [aerobically on TSA plates (E. faecalis) for 24 h and anaerobically (mixed plaque bacteria) on blood agar plates for 72 h] and observed for delayed growth after 48 h and two weeks, respectively. The percentage of killed bacteria was determined as the difference of the percentage of living cells in the initial inoculum and after exposure to the medicaments. All experiments were performed in triplicate.

4.2.2.2 Evaluation of Carry-Over Effect

Previous studies of the antibacterial effect of MTAD showed that carry-over of MTAD prevented the growth of E. faecalis on TSA plates down to the dilution of 1:1,000 (Portenier et al. 2005, Pappen et al. 2010). To determine the level of the carry over effect by the medicaments in the present study, the following control tests were done. Ten μl (for E. faecalis controls) or 100 μl (for plaque controls) samples were taken from the 500 μl mixture of 50 μl sterile water in 450 μl of each medicament and serially diluted10-fold in 990 μl and 900 μl of inactivator (first and second dilution tube) and BHI broth (third, fourth and fifth dilution tube). 10³ bacteria were then added to each dilution tube. Twenty μl from the dilutions were cultured aerobically (E. faecalis) or anaerobically (mixed plaque bacteria) as previously described. The low number of added bacteria (10³) made it feasible to detect possible differences in the growth on the plates in case of carry-over of the medicament effect. Mild or no carry-over was observed for QMiX, 1% NaOCl and 2%
NaOCl as the added bacteria grew equally well from all five dilution tubes. CHX carry over caused growth inhibition of plate cultures from the first dilution of mixed plaque bacteria, while MTAD carry over prevented growth of mixed plaque bacteria from the first two dilutions of mixed plaque bacteria and first dilution of *E. faecalis*. Because the control experiments demonstrated that carry over was a confounding factor with MTAD and CHX and could give false negative results (regarding growth), additional control experiments were performed as follows: 10 μl samples were taken from the dilution tubes and transferred into 10 ml of BHI broth each, giving a final dilution ratio of 1:10,000 from the original medicament-bacteria solution. As liquid culture does not allow colony counting, only semi quantitative analysis could be done based on the number of dilution tubes where growth could be detected. All tests were done in triplicate.

4.2.2.3 Biofilms

Sterile hydroxyapatite discs (9.6 mm diameter by 1.5 mm thickness; Clarkson Chromatography Products) and dentine discs (2 mm thickness) were used as biofilm substrate. HA discs were placed in 24-well plate (Costar) containing 2 ml of dermal type I collagen (10 μg/ml collagen in 0.012 N HCl in water; Cohesion) for overnight coating at 4°C (Shen et al. 2009). Dentin discs were obtained by cutting the tooth perpendicularly using a low-speed diamond saw (Isomet Buehler, Lake Bluff, IL, USA) after removing the crown and apical third of the tooth. *E. faecalis* strains grown overnight on TSA plates or pooled plaque from the three volunteers was suspended in BHI broth. Collagen coated HA discs and dentin discs were placed in the wells of a 24-well tissue culture plate containing 1.8 ml of BHI and 0.2 ml of either *E. faecalis* or plaque suspension in BHI was added to
each well. The discs were incubated under anaerobic conditions (AnaeroGen, OXOID) at 37°C for 21 days. BHI medium was changed once a week.

After 21 days of biofilm growth, the discs were rinsed twice in 1 ml of 0.85% physiological saline for 1 min to remove the culture broth and immersed in 1 ml of the antibacterial solutions for 1 and 3 min. Sterile water was used as a negative control. Three discs were examined for each group; five random areas of the biofilm on each disc were scanned. LIVE/DEAD BacLight Bacterial Viability stain (kit L-7012, Molecular Probes) for microscopy and quantitative assays containing separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) in solution was used for staining of the biofilm. Fluorescence from the stained cells was evaluated using a confocal laser scanning microscope (Nikon Eclipse C1, Nikon Canada). Live bacteria were observed as green while those with damaged membranes were stained red. The mounted specimens were observed using 10 x lenses with a numerical aperture of 0.30. CLSM images of the biofilms were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon) at a pixel resolution of 2.5 μm and a field resolution of 512 x 512 pixels. Each stack had a field area of 1.64 mm². The pictures were taken with a 0.5 μm step size. CLSM LIVE/DEAD images were analyzed and quantitated using the Bioimage_L software (Chávez de Paz, 2009) (available from http://www.bioimageL.com/get_bioimage_L for 2-D analysis). A 3-D analysis was done with the extended version of Bioimage_L kindly provided by Dr. Luis E. Chávez de Paz. The proportion of red fluorescence (dead cells) to green-and-red fluorescence (dead and live cells) indicated the percentage of dead cells in biofilm.
4.2.3 Data Analysis

Each solution and each time of exposure was considered an experimental group. The results from the killing tests were analyzed using one-way analysis of variance and post hoc Tukey test (SPSS version 11.5).

4.3 Results

4.3.1 Direct Exposure Test (Planktonic Bacteria)

QMiX and 1% NaOCl killed *E. faecalis* completely in 5 sec, while 2% chlorhexidine and MTAD could not eliminate all bacteria even after 3 min of exposure. There was a difference in sensitivity of the two *E. faecalis* strains to 2% CHX and MTAD. Strain VP3-181 was more sensitive than strain GEL 31 (Table 8). All tested medicaments killed mixed plaque bacteria from the three volunteers in 3 min, with QMiX and 1% NaOCl showing the fastest killing (Table 9). The carry-over effect by CHX and MTAD prevented growth of bacteria on the plates from the first dilutions. Therefore, the bacteria-medicament mixture from each dilution was further 10,000 fold diluted. Bacterial growth was detected down to the fourth dilution after 5 sec and the third dilution after 30 sec of exposure to 2% CHX or MTAD showing that after 5 sec at least 10% of bacteria survived while after 30 sec at least 1% of bacteria were still alive.
4.3.2 Biofilm Bacteria

QMIX and 2% NaOCl were the most effective medicaments at all tested times against *E. faecalis* and mixed plaque bacteria (Table 10). QMiX killed about two and four times more biofilm bacteria than 1% NaOCl and 2% CHX and up to almost twelve times more than MTAD at 1 and 3 min, respectively. QMiX killed plaque bacteria faster than *E. faecalis*. 2% NaOCl was almost as effective as QMiX against plaque biofilm bacteria, while MTAD was the least effective (Table 10). There was no significant difference in killing biofilm bacteria grown on CHA or dentin discs (p>0.05). However, all tested agents showed a tendency to kill more biofilm bacteria on CHA than on dentin discs. One possible reason is that dentin chemically inactivated the disinfecting agents. One and two percent sodium hypochlorite destroyed *E. faecalis* biofilms so quickly that CLSM data could not be obtained.
Table 8. Percentage (% ± S.D.) of killed *E. faecalis* in planktonic culture after exposure to the medicaments. CFU counted on TSA plates

<table>
<thead>
<tr>
<th>Medicaments</th>
<th>5 sec</th>
<th>30 sec</th>
<th>3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VP3-181</td>
<td>GEL 31</td>
<td>VP3-181</td>
</tr>
<tr>
<td>QMiX</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2%CHX</td>
<td>16.48±3.96</td>
<td>0</td>
<td>23.00±6.41</td>
</tr>
<tr>
<td>MTAD</td>
<td>0</td>
<td>0</td>
<td>15.43±1.33</td>
</tr>
<tr>
<td>1% NaOCl</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 9. Percentage (% ± S.D.) of killed mixed plaque bacteria in planktonic culture after exposure to the medicaments. CFU counted on blood agar plates

<table>
<thead>
<tr>
<th>Medicaments</th>
<th>5 sec</th>
<th>30 sec</th>
<th>3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plaque 1</td>
<td>Plaque 2</td>
<td>Plaque 3</td>
</tr>
<tr>
<td>QMiX</td>
<td>99.99±0.01</td>
<td>99.99±0.02</td>
<td>99.99±0.01</td>
</tr>
<tr>
<td>2%CHX</td>
<td>94.77±2.72</td>
<td>97.75±2.01</td>
<td>90.67±1.7</td>
</tr>
<tr>
<td>MTAD</td>
<td>&lt;90</td>
<td>&lt;90</td>
<td>&lt;90</td>
</tr>
<tr>
<td>1% NaOCl</td>
<td>100</td>
<td>100</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not done
Table 10. Percentage (mean ± S.D.) of dead bacteria in the biofilm exposed to different medicaments. Viability staining and CLSM

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Medicaments</th>
<th>Hydroxyapatite discs</th>
<th>Dentin discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. faecalis</td>
<td>plaque</td>
</tr>
<tr>
<td>Sterile water</td>
<td></td>
<td>1 min</td>
<td>3 min</td>
</tr>
<tr>
<td>QMiX</td>
<td>13±5.29&lt;sup&gt;a,b&lt;/sup&gt; 10.67±5.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.65±0.38</td>
<td>1.67±0.58</td>
</tr>
<tr>
<td>2% CHX</td>
<td>3±1.73      9.67±4.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.33±7.02</td>
<td>15±4.58</td>
</tr>
<tr>
<td>MTAD</td>
<td>1.67±1.15   2.67±0.58</td>
<td>4.33±3.21</td>
<td>5.67±2.08</td>
</tr>
<tr>
<td>1% NaOCl</td>
<td>N.D.        7.64±4.4</td>
<td>N.D.</td>
<td>25.72±9.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% NaOCl</td>
<td>N.D.        23.38±9.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>N.D.</td>
<td>50.03±15.94&lt;sup&gt;c,f,g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p<0.05 vs. MTAD, <sup>b</sup> p<0.05 vs. 2% CHX, <sup>c</sup> p<0.001 vs. MTAD, <sup>d</sup> p<0.001 vs. 2% CHX, <sup>e</sup> p<0.01 vs. 1% NaOCl, <sup>f</sup> p<0.01 vs. MTAD, <sup>g</sup> p<0.01 vs. 2% CHX
4.4 Discussion

This *in vitro* study aimed to evaluate the antibacterial effect of a novel irrigating solution, QMiX. *E. faecalis* and mixed plaque bacteria in planktonic culture and in biofilms were exposed to QMiX, 2% CHX, MTAD, and 1% and 2% NaOCl.

The antibacterial effect of the solutions was studied using two different experimental designs. Many of the *in vitro* and *ex vivo* studies of the antimicrobial properties of various endodontic disinfecting agents can be criticized for not reflecting well enough the *in vivo* situation in the root canal. However, in clinical *in vivo* studies many confounding factors can be present, such as variations in anatomy of the root canal and differences in the quality, quantity and location of the microbiota. Therefore, despite recognized shortcomings, well designed *in vitro* studies can provide valuable information about the comparative efficacy of the antimicrobial agents. Knowing that bacteria in nature are mostly present in biofilms, evaluation of the antibacterial effect of irrigants against biofilm bacteria should be preferred over studying the effect on planktonic cultures. The resistance to antimicrobial agents can be 100 to 1,000 fold greater for microbes in a mature biofilm compared to the same species grown planktonically (Ceri et al. 1999). On the other hand, when interpreted with great caution, planktonic killing tests do provide information about the performance of the medicaments in direct contact with bacteria, free of confounding factors (Pappen et al. 2010). Therefore, planktonic killing tests can have a role in e.g. preliminary screening of disinfecting agents. The results of planktonic killing tests have in fact been shown to have predictive value for the comparative efficacy of the same compounds against biofilm microorganisms (Pappen et al. 2010). In the present study,
QMiX and 1% NaOCl were superior to 2% CHX and MTAD in planktonic killing and successfully eliminated all *E. faecalis* and almost all mixed plaque bacteria in 5 sec.

QMiX is a new irrigating solution that contains EDTA, chlorhexidine, and a detergent (surface active agent). Its pH is slightly above neutral. It is recommended as final rinse after NaOCl. Introduction of combination products, such as MTAD or QMiX as the final irrigants after NaOCl, instead of EDTA and NaOCl is based partly on reports of dentin erosion when NaOCl is used after demineralising agents (Baumgartner & Mader 1987, Qian et al. 2011) and partly to simplify the treatment (the antibacterial and smear layer removal agent together in one solution). When the smear layer has been removed, it is usually recommended to complete the irrigation process with another disinfecting rinse to attack the remaining bacteria. The combination products provide that possibility without risk of dentin erosion which is observed when sodium hypochlorite is used again after EDTA as a final rinse (Qian et al. 2011).

The rationale for including a surface active agent in QMiX is to lower surface tension of the solution and increase its wettability (Giardino et al. 2006). Also, it may enable better penetration of the irrigant in the root canal (Abou-Rass & Patonai 1982). In addition, many surface active agents have direct antibacterial activity (cetrimide and other quaternary ammonium compounds). They target the bacterial cell membrane and cause its disorganization. Further, they provoke leakage of intracellular low-molecular weight material, degradation of proteins and nucleic acids and eventually cell wall lysis. Recently, mixtures of a surface active agent and a traditional irrigant such as CHX-Plus (chlorhexidine), Chlor-Xtra (sodium hypochlorite) or Smear-Clear (EDTA) have become
available. Several studies have indicated improved performance by the new products as compared to the same compounds without the surfactant (Shen et al. 2009, Stojicic et al. 2010, Dunavant et al. 2006). Chlorhexidine has been known for a long time for its antibacterial activity (Delany et al. 1982, Gomes et al. 2001, Manzur et al. 2007). An additional interesting feature of CHX is the additive effect with various compounds, such as hydrogen peroxide (Heling & Chandler 1998, Steinberg et al. 1999) or cetrimide (Önçag et al. 2006, Portenier et al. 2006, Arias-Moliz et al. 2010). Another potential benefit for including CHX in the mixture is its ability to adsorb onto dentin and prevent microbial colonization on the dentin surface (Ferretti et al. 1990). Even though EDTA does not have antibacterial effect on its own, it can weaken the structural integrity of the microbial cell wall and increase its permeability. The mechanism includes chelating and removing divalent cations (Mg$^{2+}$ and Ca$^{2+}$) from the bacterial cell membrane followed by another change, perhaps loss or alteration of material in the envelope (LPS) (Leive 1974, George et al. 2009).

It has been previously shown that depending on the detailed experimental design, many medicaments, including MTAD, can have a carry-over effect. In case of MTAD, this is most likely caused by tetracycline (Portenier et al. 2005, Pappen et al. 2010). The carry-over effect of a medicament can be defined as the inhibition of bacterial growth in vitro which is not due to the inhibition of growth or killing of the microbes in vivo but, rather, to the presence of high medicament concentrations not only in the test tubes during the experiment but also in the growth medium used to measure the number of microbes that survived the medicament exposure (Lounis et al. 2008). When the medicament is "carried" over to the culture medium, together with those microbes which in fact are still viable, the
medicament can prevent the growth of the residual microorganisms on e.g. the agar plate because bacteriostatic concentrations (preventing the growth) can be much lower than bactericidal concentrations (killing the microbes). A carry-over effect can occur particularly when antibiotics are used in high concentrations, which is common in endodontics and in other locally used preparations, e.g. for eye and ear infections. In the present study, all medicaments were tested separately to mitigate the possibility of the carry-over effect. Similarly to results from an earlier study (Portenier et al. 2005), MTAD suppressed bacterial growth even when 1,000 fold diluted, while 2% CHX showed only a moderate carry-over effect. QMiX showed a slight or no carry-over effect and 1% and 2% NaOCl did not show any carry-over effect when culturing was done either in BHI broth or on BHI and blood agar plates.

In the biofilm experiments, QMiX and 2% NaOCl killed about two to almost twelve times more biofilm bacteria in one and three minutes than 1% NaOCl, 2% CHX and MTAD. 2% NaOCl was more effective than QMiX at 1 min against plaque biofilm bacteria, but at 3 min QMiX (65.33%) killed more bacteria than any other solution, although the difference to 2% NaOCl (50.03%) was not statistically significant. Interestingly, the killing of *E. faecalis* in biofilm could not be measured by viability staining and CLSM as the biofilms deteriorated very quickly when exposed to hypochlorite. This indicates that there was a major difference between the mixed bacterial biofilm and *E. faecalis* biofilm. Although the reason for the different reaction to hypochlorite remains unclear, one possibility is that even after three weeks of biofilm maturation, the overall structure of the monospecies biofilm (*E. faecalis* alone) was weaker than that of a multispecies biofilm and the *E. faecalis* biofilm structure/chemistry did not offer protection against sodium hypochlorite. A recent
study by Ma et al. (2011) showed QMiX to be as effective as 6% sodium hypochlorite against *E. faecalis* biofilm in dentinal tubules.

The difference in results obtained in the current study and one by Clegg et al. (2006) who evaluated the effectiveness of sodium hypochlorite, CHX and MTAD in killing biofilm bacteria after 15 min of exposure could be explained by different experimental conditions. In Clegg’s study the biofilms were seven days old whereas in the current study biofilms were three weeks old. Also, the exposure time was longer in Clegg’s study than in the current study. Similarly, Pappen et al. (2010) tested the efficacy of MTAD, Tetraclean and five experimental solutions against two week-old biofilm bacteria. Shen et al. (2011) recently showed that sensitivity of biofilm bacteria to CHX treatment considerably changes between two and three weeks of growth which can explain the difference in results obtained from the current studies and studies by Clegg et al. (2006) and Pappen et al. (2010). Moreover, different software was used for the analysis of the results in the current study.

4.5 **Conclusion**

In conclusion, QMiX and NaOCl demonstrated higher antibacterial activity against *E. faecalis* and mixed bacterial growth in both planktonic culture and in biofilm than 2% CHX and MTAD.
Chapter 5: General Discussion and Future Directions
5.1 General Discussion

The goal of endodontic treatment is to prevent or treat apical periodontitis (Ørstavik & Pitt Ford 2008). Endodontics includes the differential diagnosis and management of oro-facial pain, pulp therapy to preserve the health of the pulp, root canal treatment, root canal retreatment, and surgical endodontics. Investigations in the 1960’s confirmed that bacteria and infection of the root canal are essential for apical periodontitis to occur (Kakehashi et al. 1965). In the 1970’s and 1980’s several classical studies improved the understanding of the microbiology of infected root canals (Sundqvist 1976, Möller et al. 1981) and the ways in which irrigants, agitation of irrigants, and medicaments can clean and disinfect the root canal (Byström & Sundqvist 1981, 1983, Moorer & Wesselink 1982). Over the past two decades our knowledge of endodontic diseases has expanded in a number of areas i.e. the role of microbial biofilms in disease etiology, causes of persistent infection, and factors that influence the outcome of endodontic treatment.

The accuracy of detection and identification of microbes in endodontic infections has also continued to improve. It was traditionally thought that there were up to 12 species of bacteria in an untreated endodontic infection (Fabricius et al. 1982). Contemporary culture-independent molecular techniques have revealed a more diverse microflora with up to 20 bacterial species per untreated endodontic infection (Munson et al. 2002). It has also been shown that the microflora of endodontically treated teeth with a persistent infection differs from that of untreated infected teeth, with E. faecalis (Molander et al. 1998), other gram-positive bacteria (Chávez De Paz et al. 2003) and C. albicans (Waltimo et al. 1997), being found more frequently in post-treatment endodontic infections.
In endodontics, biofilms have been visualised on the wall of root canals, within accessory canals (Nair et al. 1987, Siqueira et al. 2002, Ricucci & Siqueira 2010b), at the apical foramen (Nair et al. 2002) and within isthmuses (Nair et al. 2005). Biofilms usually contain multiple species, with each species contributing with their specific activities, including adaptive survival mechanisms such as resisting adverse environmental changes, host defences and antimicrobial agents. The key strategies in contemporary endodontics are aimed at disrupting and killing these well adapted microbial communities in order to eradicate all bacteria from the root canal system and to allow healing of apical periodontitis.

The research presented in this thesis expands our knowledge and understanding of dental biofilms and may help to develop more effective treatments for their elimination. More specifically, a new, Shen et al. (2009) multispecies biofilm model was introduced with a closely defined timeline of biofilm development which contains both sensitive and resistant phases of maturation. Further, using this biofilm model, the antimicrobial efficacy of conventional photoactivated disinfection was evaluated, and an improved, modified photoactivated disinfection strategy was developed. Finally, the antibacterial efficacy of a novel endodontic irrigant, QMiX, was assessed in comparison with existing disinfecting strategies in endodontics using the new multispecies biofilm model.

The most significant findings of my research can be summarized as follows:
It has been known for a long time that pure cultures of bacteria rarely exist in the real world. However, the majority of studies of bacteria and disinfection agents have been done using planktonic cultures only. The difference in susceptibility to antimicrobial agents between bacteria grown in biofilms and bacteria cultured planktonically can be considerable; free-floating bacteria are 100 to 1,000 fold more sensitive than the same species grown in biofilms (Ceri et al. 1999). In order to obtain results with better relevance for the clinical endodontic work, it is imperative to develop multispecies biofilm models for evaluation of endodontic disinfecting agents. Knowledge of the biofilm characteristics is necessary for the development of more effective strategies for biofilm elimination (ten Cate 2006). Since there are a variety of biofilm models each of which employ unique growth conditions and different substrate surfaces it is often impossible to compare the results from these studies or to interpolate them to the clinical situation. During the last few years our group has worked to develop a standardized biofilm model that could simulate some key aspects of the \textit{in vivo} biofilms and make it possible to compare results from different studies done at different laboratories, and eventually help to improve the effectiveness of anti-biofilm strategies in the clinical practice (Shen et al. 2009, 2010a, 2010b, 2011). In my research, the multispecies \textit{in vitro} biofilm model was structurally examined using transmission electron microscopy. The effect of the source of biofilm bacteria and biofilm age on the susceptibility of the biofilm to a variety of different antibacterial agents was evaluated. The results suggest that this \textit{in vitro} biofilm model shares some interesting features with \textit{in vivo} oral biofilms. Also, the level of biofilm maturation is one of the most
important characteristics associated with the sensitivity and resistance of biofilm bacteria to antibacterial agents. Bacteria in young biofilms were more sensitive to disinfectants than bacteria in mature biofilms. The change in biofilm sensitivity to the tested antibacterial agents occurred between two and three weeks of growth irrespective of the source of biofilm bacteria and for all disinfecting agents used. After three weeks, no changes were detected in the level of resistance to antimicrobial agents. These results emphasize the importance of knowing and reporting the level of maturation (time line of the development of resistance) of the biofilm model used in different studies of the antibacterial efficacy of the disinfecting agents. The knowledge of the timing in different models of such key phases of biofilm development is important also for other type of biofilm research, e.g. when studying changes in quorum-sensing patterns in the biofilms just before, during, and after the change from a sensitive to a resistant biofilm.

5.1.2 Additive/Synergistic Effects of Disinfecting Agents on Photodynamic Therapy

One of the important requirements of an ideal irrigant is its ability to eliminate microorganisms from the root canal system. The antimicrobial effect can be a direct chemical effect or an indirect effect by facilitating the mechanical disinfection through lubrication, tissue dissolution, and flushing of contaminated debris accumulated during root canal preparation. Optimally, root canal irrigants should be biocompatible with oral tissues. The most commonly used endodontic irrigant is sodium hypochlorite (Whitten et al. 1996). It is anti-microbial and effective against most bacteria (Byström & Sundqvist 1983). However, it is also toxic to surrounding tissues (Pashley et al. 1985). The search for an ideal root canal irrigant continues with the development of new materials and methods.
Photoactivated disinfection was introduced to dentistry as a safe method for the elimination of microorganisms in periodontal and endodontic infections. However, several studies have shown that the efficacy of PAD is inferior to other common endodontic irrigants such as sodium hypochlorite (Soukos et al. 2006, Bergmans et al. 2008). In the present study the PAD was developed further and improved by optimising the concentration of the photoactive agent using the new biofilm model as the test environment, and by combining the action of low concentrations of common disinfectants and PAD. One of the most important findings of the study was that the photosensitizer methylene blue at its most effective concentration for the killing of planktonic bacteria (15 µM) failed to have almost any effect on biofilm bacteria. Experiments with a wide range of different MB concentrations showed that the most effective MB concentration against biofilm bacteria was almost seven times higher (100 µM) than the 15 µM used for PAD on planktonic cells. Interestingly, increasing the MB concentration over 100 µM reduced the PAD effect rapidly. The study further demonstrated that the addition of low concentrations of endodontic irrigants such as EDTA, CHX and hydrogen peroxide to methylene blue resulted in a threefold reduction in the time needed to kill the planktonic bacteria. Generally, the same trend was observed in PAD killing of biofilm bacteria. After adjusting the concentration of MB and adding low concentrations of EDTA, CHX and H₂O₂ up to almost twenty two times more biofilm bacteria were killed than by conventional PAD, and up to almost eight times more bacteria were killed than by traditional endodontic irrigants (2% CHX, 1% and 2% sodium hypochlorite). These data suggest that careful adjustment of the concentration of photosensitizer and additive/synergistic action of low concentrations of disinfecting agents and PAD may have great promise in endodontic treatment.
Irrigation has a central role in endodontic treatment. During and after instrumentation, irrigants assist removal of dentin chips, tissue remnants, and microorganisms from the root canal. Irrigants can also help prevent packing of the hard and soft tissue in the apical portion of the root canal as well as extrusion of infected material into the periapical area. Several irrigating solutions have antimicrobial activity and actively kill bacteria and yeasts when introduced in direct contact with the microorganisms. In addition, some irrigating solutions have the ability to dissolve either organic or inorganic tissue in the root canal. On the other hand, various irrigating solutions can also have tissue toxicity and may cause severe pain if they gain access into the periapical tissues (Hülsmann & Hahn 2000). Therefore, an optimal irrigant should have all or most of the positive characteristics such as washing action, reducing instrument friction during preparation, dissolving inorganic tissue and organic matter, penetrating to canal periphery, killing bacteria and yeasts but not irritate or damage vital periapical tissue or weaken tooth structure (Walton & Torabinejad 1989, Zehnder 2006, Haapasalo et al. 2010). None of the available irrigating solutions can fulfill all these criteria. For optimal effect, a combination of two or three irrigants in a correct irrigation sequence should be used for a successful treatment outcome.

In the past decade, much effort has been put on improving currently available endodontic irrigants and protocols or developing new irrigants for more efficient elimination of bacteria and debris from the root canal. Recently, mixtures of a surface active agent and a traditional irrigant such as CHX-Plus (chlorhexidine), Chlor-Xtra (sodium hypochlorite) or Smear-Clear (EDTA) have become available on the market. Several studies have indicated...
improved performance by the new products as compared to the same compounds without the surfactant (Shen et al. 2009, Stojicic et al. 2010, Dunavant et al. 2006). In the present study the efficacy of a new endodontic irrigant QMiX against bacteria in planktonic and biofilm culture was tested. QMiX is combination product that contains chlorhexidine, EDTA and a surfactant. It was demonstrated that QMiX was more efficient in killing planktonic and biofilm bacteria than 2% chlorhexidine, BioPure MTAD and 1% and 2% sodium hypochlorite. An additional outcome of this study was the demonstration that the novel in vitro biofilm model is a promising tool for assessing the antibacterial effectiveness of endodontic irrigants.

5.2 The Impact of the Thesis

The findings of this thesis carry both research and clinical significance. Some of the ways in which this work advances understanding of the dynamics of killing biofilm bacteria are described below:

- This work investigated a new, Shen et al. (2009) in vitro multispecies biofilm model developed to mimic in vivo biofilms. Morphological and functional similarities between this in vitro biofilm and true in vivo biofilms as described in the literature suggest that this model can be successfully used in endodontic research. Also, the biofilm maturation timeline demonstrated in an earlier study (Shen et al. 2011) for one mixed bacterial biofilm has now been confirmed in this thesis for six different biofilms. Furthermore, it has been shown for the first time that susceptibility of biofilm bacteria to disinfecting agents with different mechanisms of action is more
dependent on biofilm maturation and less dependent on the source of biofilm bacteria. The work completed and published will fill a knowledge gap in the literature and stand as a guide to researchers who wish to study biofilms and strategies for biofilm elimination.

- This work has provided a re-evaluation of photoactivated disinfection and introduced an advanced, modified PAD protocol that has been shown to be almost twenty two times more effective than conventional PAD in killing biofilm bacteria. A novel approach to this irrigation protocol has the potential to contribute both to endodontic research and clinical work. The combination of two or more irrigants in low concentrations and their synergistic and/or additive effect has been found to be an efficient way of achieving a more effective killing of biofilm bacteria with little or no harm to host tissue. The published findings from this work will serve as a model that can be applied to other disinfecting protocols and/or irrigants. Modified PAD itself could have significant clinical application in endodontic treatment.

- A novel *in vitro* biofilm model has been used to test new commercially available endodontic irrigant QMiX (a combination of EDTA, CHX, and a detergent). There are two important achievements from this study both for research and clinical treatment. Firstly, this work adds to the evidence that a new *in vitro* multispecies biofilm model is suitable for testing existing as well as new endodontic irrigants. Excellent results by QMiX in the killing of biofilm bacteria suggest QMiX as an effective endodontic irrigant for clinical treatment. This work is also published;
therefore researchers can benefit from the unique features of this biofilm model to evaluate the antibacterial efficacy of available endodontic irrigants or to develop new ones.

5.3 **Future Directions**

A number of studies should be carried out to expand the findings of this thesis. The research should include:

1. Analysis of the effect of environmental factors such as oxygen level and the type and availability of nutrients on the speed of biofilm maturation and the susceptibility of biofilm bacteria to antimicrobial agents.

2. Identification of the specific species of bacteria present in this biofilm model (both young and mature) using molecular biological methods such as pyrosequencing and specific primers, and perform a population analysis in comparison to bacteria found in *in vivo* endodontic and other oral biofilms.

3. Perform an analysis of the amount, development and composition of extracellular polymeric substances and the role these three factors play in the resistance of biofilm bacteria to antibacterial agents.

4. Evaluation of other biofilm models and their possible use for assessing the antibacterial efficacy of antibacterial agents.
5. Research into a deeper understanding of the similarities and differences between monospecies and multispecies biofilms, and the susceptibility/resistance of bacteria in these two types of biofilms to antibacterial agents.


7. Assessment of photoactivated disinfection (conventional and modified) using various biofilm models.
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Appendices

Appendix A

A.1 Focused Ion Beam (FIB) SEM Analysis of *In vitro* Biofilm Model

The results presented in Appendix A are preliminary results of an *in vitro* biofilm examination obtained by focused ion beam scanning electron microscopy (FIB SEM).

FIB SEM images demonstrated a variety of bacterial morphotypes; cocci (a) and rods (b) were the most frequent (Figure 13A, Figure 13B). Among bacterial cells, there was visible empty space that most likely corresponded to biofilm water channels (c). However, dense material that was noted between bacterial cells most likely corresponded to the extracellular polymeric substance (d).
Figure 13. FIB SEM image of a 3-week-old in vitro biofilm. (A) a- coccus, b- rod, c- water channel, d- extracellular polymeric substance (B) a- coccus, c- water channel
Appendix B

B.1 Confocal laser scanning microscopy and the analysis of killed biofilm bacteria

Biofilms exposed to the tested agents/protocols were stained using LIVE/DEAD BacLight Bacterial Viability stain (kit L-7012, Molecular Probes; SYTO 9 and propidium iodide in 1:1 mixture). A drop of 80 µl of the stain mixture was placed on the top of each biofilm. Stained biofilms were scanned using confocal laser scanning microscope (Nikon Eclipse C1, Nikon Canada; 10 x lenses and a numerical aperture of 0.30). Five random areas were scanned (Figure 14) on each disc and each area was scanned through the whole biofilm thickness (Figure 15). CLSM images of the biofilms were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon). Pixel resolution was 2.5 µm and a field resolution was 512 x 512 pixels. Each stack had a field area of 1.64 mm². The step size of the scans was 0.5 µm. About one hundred twenty images were acquired of each stack. CLSM images were analyzed and the number of killed bacteria quantified using bioImage_L software (Chávez de Paz, 2009) (available from http://www.bioimageL.com/get_bioimage_L. for 2-D analysis). 3-D analysis was done using the extended version of bioImage_L kindly provided by Dr. Luis E. Chávez de Paz (Figures 16, 17). The proportion of red fluorescence (dead bacteria) to green-and-red fluorescence (dead and live bacteria) indicated the percentage of dead cells in biofilm.
Figure 14. Five random areas of biofilm (stratified sampling) chosen to be scanned by CSLM
Figure 15. A biofilm area to be scanned through the whole thickness of the film
Figure 16. *BioImage_L*, a software for the analysis of LIVE/DEAD biofilm images
Figure 17. The analysis of LIVE/DEAD biofilm images using BioImage_L (scale= 2.5 µm/pixel, z-step= 0.5 µm, noise ratio= default)