Comparative Efficacy Of Endodontic Medicaments Against *Enterococcus Faecalis* Biofilms

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Abstract

It is well established that bacteria cause pulpal and periradicular disease (Kakehashi et al. 1965). Of the bacteria recovered from failing root canals, Enterococcus faecalis is one of the most prevalent species (Molander et al. 1998; Sundqvist et al. 1998). Many laboratory studies have investigated the effectiveness of root canal irrigants and medicaments against E. faecalis. Most used planktonic cultures, which are not representative of the in vivo growth conditions of an infected root canal system, where bacteria grow as a biofilm adhering to the dentinal wall (Nair 1987). Organisation of bacteria within biofilms confers a range of phenotypic properties that are not evident in their planktonic counterparts, including a markedly reduced susceptibility to antimicrobial killing (Wilson 1996).

Objectives: The aims of this study were: 1) To compare the efficacy of commonly used endodontic medicaments against E. faecalis cultured as a biofilm. The medicaments tested were Ledermix paste, calcium hydroxide, Odontopaste, 0.2% chlorhexidine gel and 50:50 combinations of Ledermix/calcium hydroxide and Odontopaste/calcium hydroxide. 2) To compare the antimicrobial effect achieved through exposure to endodontic medicaments with that achieved by exposure to a constant concentration of sodium hypochlorite for varying times.

Methods: A biofilm was established using a continuous flow cell. E. faecalis inoculum was introduced into the flow cell and allowed to establish on human dentine slices over
4 weeks. Each test medicament was introduced into the flow cell for a period of 24 or 48 hours, while sodium hypochlorite was evaluated after 1, 10, 30 and 60 minutes. Biofilms were harvested by sonication in sterile PBS. Cellular protein levels were measured to quantitate the amount of biofilm harvested.

Cellular viability was determined using serial plating. The number of colony forming units was then adjusted for cellular protein levels to allow treatment protocols to be compared. Qualitative SEM analyses of the biofilm was performed following exposure to each test agent.

**Results:** Sodium hypochlorite was the only agent that achieved total bacterial elimination. Ledermix and Odontopaste had no significant effect on the *E. faecalis* biofilm, while calcium hydroxide and 50:50 combinations of calcium hydroxide with either Ledermix or Odontopaste were able to reduce viability by > 99%.

**Conclusion:** When used in isolation, antibiotic containing medicaments had no appreciable effect on the viability of *Enterococcus faecalis*. Sodium hypochlorite remains the gold standard for bacterial elimination in root canal therapy.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Barbara Plutzer
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1. Literature Review

1.1 Introduction

In 1894 W.D. Miller described with fascination the micro-organisms seen in and recovered from the infected root canal. He was perhaps the first to associate disease and inflammation in the jaws with the infected pulp canal space (Miller 1894). Since that time, the microbiological basis of both endodontic disease and of endodontic treatment failure has become firmly established (Kakehashi et al. 1965; Sjögren 1997). Bacterial penetration of the pulp is often asymptomatic but may lead to pain and discomfort, and it is the sequel of infection that inspires greater concern and provides the strongest impetus for intervention. Whereas the common, chronic forms of apical periodontitis seldom pose medical problems of any magnitude, it is the smaller number of cases that spread to cause inter-fascial infections and possibly serious complications at distant sites that form a back-drop on which the concepts and rationales for treatment are based (Ørstavik 2003).

1.2 Microbiological goals of Endodontic Treatment

Apical periodontitis is described as an infectious disease caused by microorganisms colonizing the root canal system (Kakehashi et al. 1965; Sundqvist 1976; Möller et al. 1981). The endodontic treatment of teeth with irreversibly inflamed pulps is essentially a prophylactic treatment from a microbiological perspective, because the radicular vital pulp is usually free of infection; and the rationale is to prevent further infection of the
root canal system and the emergence of apical periodontitis (Haapasalo 2005). In cases of infected necrotic pulps or in root canal-treated teeth associated with apical periodontitis of infective origin, an intraradicular infection is already established, and the goal of endodontic procedures becomes the elimination of the infecting microorganisms. Ideally, endodontic treatment procedures should sterilize the root canal, but given the complex anatomy of the root canal system, it is widely recognized that, with available instruments and techniques, fulfilling this goal is utopic for most cases. The reachable goal is to reduce bacterial populations to a level below that necessary to induce or sustain disease (Siqueira and Rôças 2008).

1.3 Endodontic success and failure

When endodontic treatment is performed under aseptic conditions and according to accepted clinical principles, the success rate is generally high. Most follow up studies on endodontic therapy report overall success rates of 85%-90% (Strindberg 1956; Seltzer et al. 1963; Kerekes and Tronstad 1979; Molven and Halse 1988; Sjögren et al. 1990). Epidemiological studies, however, report success rates of only 60-75%, identifying a surprisingly high prevalence of apical periodontitis associated with root filled teeth (Eriksen 2002).

Although many failure cases are caused by technical problems during treatment, some cases fail even when apparently well treated (Sundqvist et al. 1998). Studies investigating the aetiology of endodontic treatment failures in cases where the initial therapy was performed to a satisfactory standard have identified five factors that may
contribute to a persistent periapical radiolucency following treatment. The factors are: intraradicular infection (Nair et al. 1990), extraradicular infection especially by bacteria of the species Actinomyces israelii and Priopionibacterium propionicum (Nair and Schroeder 1984; Sjögren et al. 1988) foreign body reactions (Yusuf 1982; Nair et al. 1990); cysts, especially when containing cholesterol crystals (Nair et al. 1993) and fibrous scar tissue healing following conventional treatment (Nair et al. 1999). In most cases, failure of endodontic treatment is believed to be due to microorganisms persisting in the apical parts of the root canal system, even in seemingly well-treated teeth (Nair et al. 1990).

1.4 Enterococci

Enterococci are gram-positive cocci that occur singly, in pairs, or as short chains. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen (Gilmore 2002). Enterococcus species live in vast quantities in the human intestinal lumen and under most circumstances cause no harm to their host. They are also present in human female genital tracts and the oral cavity in lesser numbers (Koch et al. 2004). One of the characteristic features of enterococci is their ability to survive very harsh environments including extreme pH levels (4.0-11.0) (Nakajo et al. 2006) and salt concentrations. They resist bile salts, detergents, heavy metals, ethanol, azide and desiccation (Gilmore 2002). They can grow in the range of 10°C to 45°C and survive a temperature of 60°C for 30 minutes (Hartke et al. 1998).
Over the last few decades, enterococci have emerged as important nosocomial pathogens. This importance is attributed primarily to the high degree of antibiotic resistance that is exhibited by most enterococci. Of particular concern has been the rapid spread of enterococci with resistance to vancomycin (VRE), creating therapeutic problems for patients with serious infections such as endocarditis (Gilmore 2002). Enterococcus faecalis is responsible for 80% of all infections caused by enterococci, with E. faecium responsible for the remaining 20% (Ruoff et al. 1990).

1.5 Enterococci in the normal flora of the oral cavity

Enterococci form part of the normal gastrointestinal tract flora in animals and humans, though only a few studies have focused on their prevalence in the oral cavity. In an early report, Williams et al. collected saliva samples from 206 individuals and analysed them for enterococci. Saliva was collected more than once from most donors. Overall, enterococci were detected in the saliva of 21.8% of subjects on at least one occasion. However, the carriage was not consistent, meaning that on one experimental day an individual tested positive, while testing negative on a subsequent day (Williams et al. 1950). More recently, Sedgley et al. investigated the prevalence, phenotype and genotype of oral enterococci. The study used microbiological culturing methods to detect enterococcal isolates in the oral rinse samples of 11% of 100 patients receiving endodontic treatment and 1% of 100 dental students with no history of endodontic treatment. All enterococcal isolates were identified as E. faecalis (Sedgley et al. 2004). In a follow-up study using quantitative real-time polymerase chain reaction (PCR) as well as microbiological culturing, 30 oral rinse samples from endodontic patients were analysed for E. faecalis. Quantitative PCR detected E. faecalis in 17% of the samples,
while parallel culture assays were less sensitive, detecting the microorganism in only 7% of samples. Proportionally, oral *E. faecalis* comprised less than 0.005% of the total bacterial load (Sedgley *et al.* 2005).

A significantly higher prevalence of oral enterococci was detected in a recent study which used a multi-site oral sampling strategy in combination with sensitive detection methods incorporating both culture and PCR. *E. faecalis* was detected in at least one tongue, oral rinse, or gingival sulcus sample in 68% of patients and in the root canals of 5% of patients. The tongue was found to be the most prevalent detection site (Sedgley *et al.* 2006). In other studies oral *E. faecalis* was detected at a higher prevalence in periodontitis patients compared to controls (Sedgley *et al.* 2006; Souto and Colombo 2008). Evidence shows that *E. faecalis* is clearly a part of the human oral microbiota. In healthy individuals not being treated with wide-spectrum antibiotics, the relative amount of enterococci is very small, often below the detection levels of normal sampling and culturing methods (Portenier 2003). Recent publications, however, confirm former speculations that the occurrence of *E. faecalis* in the oral cavity is higher than previously suggested.

A thought provoking recent review contends that enterococci are not part of the typical commensal microbiota of the oral cavity, but rather a transient infective microorganism sourced from food. Enterococci are ubiquitous in food products, such as cheese, fermented sausages, minced beef, pork, and fish (Zehnder and Guggenheim 2009). Razavi *et al.* investigated the clearance of *E. faecalis* from a highly contaminated cheese among healthy volunteers. One minute after ingestion, a median of 5480 colony
forming units were recovered. Bacterial counts were significantly reduced after 100 minutes, and below the limit of detection after 1 week (Razavi et al. 2007). These findings may suggest that enterococci adhere to oral tissues of healthy subjects but fail to become permanently established within the oral microbiota and are thus gradually eliminated. This interpretation correlates well with Williams’ early findings of sporadic sampling of *E. faecalis* in the saliva of the same individuals.

### 1.5.1 Root canal sampling

Absorbent paper points are typically used to sample the contents of the root canal for bacterial culturing. (Möller 1966). Paper point sampling is clinically convenient. However, establishing whether the microorganisms sampled are representative of those involved in the infectious process is generally difficult. The technique also has other limitations. A sample may show the presence of microorganisms although the root canal is sterile – a false positive sample – or no microorganisms may be revealed although the root canal system is infected – a false negative sample. False positive samples are primarily caused by contamination from the oral cavity, a problem of particular significance when PCR technology is used. The risk of obtaining a false negative sample is equally as great. The root canal system is often a complex arrangement of main, lateral and accessory canals, as well as ramifications, isthmuses and cul de sacs (Vertucci 1984). These anatomical niches provide a protective environment for bacterial survival, and they are precisely the regions that are difficult to reach when taking a microbiological sample. False negative samples can also be due to limitations in the cultivation methods available, combined with the fastidious nature of the organisms. In fact, of the 620 predominant oral bacterial species, about 35% have not yet been cultured *in vitro* (Paster & Dewhirst 2009).
Despite these inherent limitations, serial sampling of root canals at various stages of root canal therapy showcases the changing microbiological environment, and provides a means of evaluating the effectiveness of various stages of therapy. Various studies have contributed to our understanding of the endodontic infection in untreated infected root canals (Sundqvist 1976; Fabricius et al. 1982; Munson et al. 2002), in root canals where therapy has been initiated (Siren et al. 1997), and in teeth with post treatment disease (Möller 1966; Molander et al. 1998; Sundqvist et al. 1998; Hancock et al. 2001).

1.5.2 Enterococci in primary apical periodontitis

Although more than 700 species of bacteria have been isolated from the oral cavity, only a limited number have been consistently isolated from endodontic infections (Sundqvist 1994). The selective pressures operating in the root canal environment, including a low redox potential and nutrients rich in peptides and low in carbohydrates, favour the strong dominance of strictly anaerobic bacteria typical of primary apical periodontitis. Together with some facultative anaerobic bacteria such as streptococci, lactobacilli and Actinomyces, they can establish a wide variety of bacterial combinations. Usually three to six species can be isolated from a single tooth using conventional culturing techniques (Sundqvist 1976; Fabricius et al. 1982). Modern molecular methods are able to detect a significantly higher number and greater diversity of species, including numerous as-yet uncultivable organisms (Munson et al. 2002). Munson et al. recovered a mean number of 20 bacterial taxa from each infected root canal sample using combined culture and molecular methods (Munson et al. 2002).
Enterococcal species have traditionally been regarded as atypical isolates in primary endodontic infections. Culturing methods of detection have isolated enterococci in 5-12% of sampled root canals (Engström 1964; Möller 1966; Sundqvist et al. 1989).

More recently, Siqueira et al. used molecular techniques to examine the prevalence of Actinomyces spp., streptococci and \textit{E. faecalis} in primary root canal infections. Samples were obtained from 53 infected teeth, of which 27 had acute periradicular abscesses. The checkerboard DNA-DNA hybridization assay detected streptococci in 22.6% of the samples, actinomyces spp. in 9.4%, and \textit{E. faecalis} in 7.5%. The occurrence of \textit{E. faecalis} was significantly lower in acute infections (3.7%) than in asymptomatic teeth (11.5%) (Siqueira et al. 2002). Rôças et al. similarly concluded that \textit{E. faecalis} was more frequently detected in asymptomatic cases. Using the nested PCR method, \textit{E. faecalis} was detected in 33% of root canals associated with asymptomatic chronic periradicular lesions, in 10% of root canals associated with acute apical periodontitis, and in 5% of the pus samples aspirated from acute periradicular abscesses (Rôças et al. 2004).

The proportions of \textit{E. faecalis} as part of the total microbial load were studied by examining samples from both primary infection and retreatment cases. The samples were divided into two equal aliquots that were independently analysed by investigators using culturing and real-time PCR methods. \textit{E. faecalis} was detected in a surprisingly high 67.5% of 40 primary infection samples and 89.6% of the 48 retreatment samples. The high prevalence was attributed by the authors to the sensitivity of the molecular method used and the patient selection which was based on referrals of teeth with suspected persistent infection. The median proportion of \textit{E. faecalis} in the samples was 2.8% of the total bacteria in the primary infection samples and 0.98% in the retreatment
It is obvious that while the prevalence of *E. faecalis* is high, particularly in retreatment cases, proportionally this microorganism makes up only a very small part of the total bacterial flora. This may imply that other organisms present did not reach detection levels, or had more fastidious growth requirements that could not be mimicked in the laboratory environment.

### 1.5.3 Enterococci in the root canal after initiation of treatment

Reports of culture reversals in longitudinal studies, characterized by the sudden positive sampling of enterococci after initiation of treatment, have led to speculations of coronal leakage through the temporary restoration (Sjögren *et al*. 1991; Sundqvist *et al*. 1998). Siren *et al.*, studied the correlation between several clinical parameters and the occurrence of enterococci in teeth where treatment did not result in healing. The clinical treatment history of 40 *Enterococcus*-positive and 40 *Enterococcus*-negative teeth was compared. A higher proportion of *E. faecalis* was found in teeth whose canals lacked an adequate seal for a period during the treatment or teeth that were treated over 10 or more visits (Siren *et al*. 1997). Compromised asepsis during endodontic treatment thus appears to be an important causative factor for contamination of the root canal by *E. faecalis*.

The possibility of *E. faecalis* from food products causing a transient oral infection of sufficient magnitude to infect the root canal was recently tested by Kampfer *et al*. An artificial oral environment was established using a masticator device perfused with artificial saliva in which test teeth, temporarily restored with Cavit, were exposed to
controlled loads of mastication over a period of 1 week. A cheese containing viable *E. faecalis* cells was placed between the occlusal surfaces of the test teeth. Of the 16 specimens restored with 2mm of Cavit, 6 showed leakage, while 1 of the 16 specimens with a 4mm cavit restoration had cultivable *E. faecalis* cells detected. These results go some way to giving credence to the suspicion that food derived microbiota could enter the root canal system via microleakage (Kampfer *et al.* 2007).

1.5.4 Enterococci in post-treatment apical periodontitis

The microbial flora of root-filled canals with persisting periapical lesions differs markedly from that of untreated necrotic dental pulps, with a very limited assortment of microorganisms able to survive. Generally, only one or a small number of species are recovered, with a predominance of gram-positive microorganisms and facultative anaerobes (Möller 1966; Molander *et al.* 1998; Sundqvist *et al.* 1998; Hancock *et al.* 2001). *E. faecalis* is the most commonly recovered species in these teeth (Siqueira and Rôças 2004; Sundqvist *et al.* 1998; Molander *et al.* 1998; Pinheiro *et al.* 2003; Gomes *et al.* 2008), with the likelihood of detection in failing root canals 9 times higher than in primary endodontic infections (Rôças *et al.* 2004).

As far back as 1964, Engström examined 223 teeth for the presence of enterococci. In teeth undergoing primary endodontic treatment, enterococci were isolated in 12% of culture positive cases, while for previously root-filled teeth, the recovery rate was 21% (Engström 1964).
Sundqvist retreated 54 teeth with post-treatment disease. Microbial growth was recovered in 24 of the canals (45%) and *E. faecalis* was the most frequent isolate, detected in 38% of culture-positive canals. On each occasion, *E. faecalis* was isolated in pure culture. The success rate for the teeth from which *E. faecalis* was isolated after removal of the earlier root filling was somewhat lower (66%) than the overall average (74%) (Sundqvist et al. 1998).

Molander examined the microbiological status of 100 root-filled teeth with apical periodontitis. The presence of intracanal microbiota was demonstrated in 68% of teeth, most of which contained one or two strains of microorganisms. *E. faecalis* was the most frequently isolated species, recovered in 32 teeth, or 47% of the culture-positive root canals. A further 20 teeth without signs of periapical pathosis were similarly cultured for microbial presence. Mostly sparse growth of intracanal bacteria was detected in 9 teeth (45%), while 11 were deemed bacteria-free (Molander et al. 1998).

Pinheiro et al. studied the microbial flora of 60 root-filled teeth with persisting periapical lesions. Microorganisms were recovered from 51 teeth (85%); mostly one or two strains were present per canal. Of the species isolated, 57% were facultative and 43% obligate anaerobes. *E. faecalis* was the most frequently recovered species, detected in 53% of culture-positive canals, 18 times in pure culture. Significant clinical associations were observed between polymicrobial or anaerobic infections and pain: *Prevotella intermedia/Prevotella nigrescens* and tenderness to percussion; *Streptococcus* spp./*Actinomyces* spp. and sinus formation; and *Streptococcus* spp./*Candida* spp. with coronally unsealed teeth (Pinheiro et al. 2003).
Siqueira & Rôças identified microorganisms associated with post-treatment disease using PCR. DNA was extracted from 22 teeth selected for endodontic re-treatment, and analysed for the presence of 19 target species. All samples were positive for at least one of the microorganisms, with *E. faecalis* being the most prevalent species and detected in 77% of teeth. The mean number of species in canals filled 0-2mm short of the radiographic apex was 3 (range 1-5), whereas canals filled shorter than 2mm from the apex yielded a mean of 5 species (range 2-11) (Siqueira and Rôças 2004). These findings support the notion that the microflora of poorly obturated root canals resembles more closely that of a primary endodontic infection.

Gomes *et al.* recently investigated the presence of selected bacterial species in 45 teeth with post-treatment disease using PCR analysis. The bacterial isolates detected in each canal were correlated with the clinical features of each case. The 9 target species were detected in 39 of 45 cases (87%). *E. faecalis* was the most frequently recovered species, detected in 78% of the study teeth, followed by *Peptostreptococcus micros* (51%), *Porphyromonas gingivalis* (36%), *Filibacter alocis* (27%), *Treponema denticola* (24%) *Porphyromonas endodontalis* (22%), *Prevotella intermedia* (11%) and *Prevotella nigrescens* (11%). *T. denticola* and *P. micros* were statistically associated with tenderness to percussion. *P. nigrescens* was associated with the presence of spontaneous pain and abscess; *P. endodontalis* and *P. nigrescens* were associated with purulent exudates (Gomes *et al.* 2008).

In summary, enterococci have been identified in a considerable proportion of teeth with persisting periapical lesions that had endodontic therapy completed to a technically
satisfactory level (Sundqvist et al. 1998), as well as in counterparts that had insufficient root fillings (Peciuliene et al. 2000). They are detected frequently in studies from different regions irrespective of treatment protocol; in root filled teeth both in regions where calcium hydroxide is commonly used as an interim dressing (Molander et al. 1998) and in countries where this topical antiseptic is usually not applied (Hancock et al. 2001).

It is apparent that prevalence data for E. faecalis are quite variable, and very much influenced by the method of detection used in each investigation. Cultivation is time-consuming, requires controlled conditions during sampling and transport to ensure microorganism viability, and can lead to variable results based on the experience of the microbiologist (Siqueira and Rôças 2005). In contrast, PCR-based detection methods enable rapid identification of specific DNA sequences with a high degree of sensitivity. This higher sensitivity, however, can be at least partly attributed to the detection of free floating DNA, and DNA from nonviable, culturable viable cells and viable but non-culturable cells (Sedgley et al. 2006). Consequently, the number of intact and viable microorganisms cannot be readily established. In addition, molecular techniques analyse total DNA extracted from the sample and in the process destroy the microorganism. Thereafter, being no longer culturable, it is not possible to study phenotypic characteristics and potential pathogenicity associated with individual strains recovered from the root canal (Sedgley et al. 2005).

A further factor affecting the validity of many investigations is the problem of contamination from saliva or plaque from the outer tooth surface. Engström highlighted
that many teeth harboring enterococci in the root canal system also show positive enterococcal growth on the outer tooth surfaces (Engström 1964). False positive results are more likely to occur when PCR technology is used (Nair 2007). Hence, a meticulous sampling technique which includes disinfection of the tooth and the access cavity with associated sterility checks of both sites is a prerequisite to yielding meaningful results (Zehnder and Guggenheim 2009). Unfortunately, relatively few studies comply with the current decontamination protocols which recommend that root canal sampling for polymerase chain reaction be preceded by sodium hypochlorite (NaOCl) rather than iodine surface disinfection (Ng et al. 2003).

The presence of microleakage, through defective coronal restorations, old temporary restorative materials, or nonrestored teeth is also likely to influence microbial findings. In the study by Pinheiro et al. the authors acknowledge that microleakage was detected in the majority of teeth (45/60) being re-treated (Pinheiro et al. 2003), as was the case with the investigation by Gomes, where 35 of the 45 teeth had defective coronal seals (Gomes et al. 2008). Consequently, proper decontamination of the access for PCR would have been exceptionally difficult if not impossible to achieve, and enterococcal DNA could have originated from sources outside the root canal.

While there has been considerable focus in the literature on the prevalence of *E. faecalis* at various stages of endodontic infection, the clinical features of an enterococcal infection have not been elucidated. Neither Pinheiro nor Gomes, who attempted to correlate the presence of the bacterial species in root canals with the clinical features of each case, could associate *E. faecalis* with any acute symptoms in
teeth with post-treatment disease (Pinheiro et al. 2003; Gomes et al. 2008). This is consistent with Siqueira’s findings that *E. faecalis* was detected more often in symptom-free teeth with primary apical periodontitis than in teeth with acute symptoms (Siqueira et al. 2002). Kaufman et al. investigated the association between *E. faecalis* and endodontically treated teeth with and without periradicular lesions. The latter group of teeth were retreated because of technically inadequate root canal therapy or suspected coronal leakage. The study found teeth with a periradicular lesion were significantly more likely to harbor bacteria, but enterococci were detected more frequently in teeth with a normal periapex (Kaufman et al. 2005).

It has been suggested that enterococci may be selected in root canals undergoing standard endodontic treatment because of low sensitivity to antimicrobial agents (Dahlén et al. 2000). Furthermore, Love postulated that the proficiency with which this bacterium invades dentinal tubules provides it protection from chemo-mechanical root canal preparation and intracanal dressings (Love 2001). Subsequently, when the opportunity arises, *E. faecalis* could be released from the tubules into the root canal space and act as a source of re-infection (Sedgley et al. 2005). Even bacteria entombed at the time of root filling could provide a long-term nidus for subsequent reinfection. In a study evaluating the survival of entombed *E. faecalis*, root canals were inoculated with one of two strains of this microorganism following chemomechanical preparation. After 48 hours of incubation, the canals were obturated with gutta percha, a zinc-oxide eugenol sealer and restored with glass ionomer cement (GIC). Culture, PCR and histological methods were used to recover *E. faecalis* after 6 and 12 months. All root filled teeth demonstrated viable *E. faecalis* cells (Sedgley et al. 2005).
An alternative possibility is that enterococci are opportunistic coronal invaders of the improperly sealed necrotic or filled root canal system, gaining entry during or after treatment (Zehnder and Guggenheim 2009). Whether *E. faecalis* is a major pathogen involved with the aetiology of endodontic failures or merely a colonizer that takes advantage of the environmental conditions within obturated root canals has yet to be fully appreciated (Rôças *et al*. 2004).

### 1.5.5 Virulence Factors

An appreciation of the potential virulence factors of *E. faecalis* is useful in our attempts to decipher the role of this microbe in endodontic infections. Enterococci possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immune-modulation and cause toxin-mediated damage (Portenier 2003). These factors are discussed below.

#### 1.5.5.1 Aggregation Substance

This is a surface localized protein, which mediates the cell-to-cell contact that enables the exchange of plasmids between recipient and donor strains. Plasmids are autonomous, covalently closed circular, double-stranded, supercoiled DNA elements, which frequently confer traits that facilitate growth and survival under atypical conditions, such as resistance to antibiotics (Sedgley *et al*. 2005). Aggregation substance may serve as a virulence determinant by assisting the dissemination of plasmid-encoded virulence factors and facilitating attachment to leukocytes, connective extracellular matrix and possibly dentine (Hubble *et al*. 2003). *E. faecalis* strains
expressing aggregation substance were able to bind to type I collagen twice as effectively as aggregation substance-negative strains (Rozdzinski et al. 2001). This may be of particular importance with respect to endodontic infections, since collagen is the main organic component of dentine (Kayaoglu and Ørstavik 2004). Hence, an expression of aggregation substance could enable the E. faecalis cells to attach and colonise the dentine surface and possibly be a factor in their invasion of dentinal tubules.

Aggregation substance may also act as a protective element, favouring bacterial survival by assisting in efforts to resist host defence mechanisms in endodontic infections. For example, aggregation substance has been reported to facilitate the intracellular survival of phagocytosed E. faecalis cells within human macrophages (Kayaoglu and Ørstavik 2004). Similarly, E. faecalis cells expressing aggregation substance have been found to be more resistant to killing by human neutrophils (Rakita et al. 1999). Aggregation substance has also been identified as being capable of inducing inflammation through the stimulation of T-lymphocytes, followed by a massive release of inflammatory cytokines, and leading to host tissue damage (Kayaoglu and Ørstavik 2004). Sedgley et al. identified aggregation substance in all endodontic enterococcal strains tested (Sedgley et al. 2005).

1.5.5.2 Enterococcus surface protein (Esp)

This is a large chromosome-encoded surface protein, whose role in virulence is yet to be fully elucidated. It is speculated, however, that Esp has a role in allowing enterococcal cells to evade the host immune system. Toledo-Arana et al. demonstrated a relationship
between the presence of Esp and the biofilm formation capacity of *E. faecalis*, with none of the esp-defective *E. faecalis* strains able to establish biofilm (Toledo-Arana *et al.* 2001).

### 1.5.5.3 Gelatinase

Gelatinase is an extracellular zinc-containing metalloproteinase capable of hydrolyzing gelatin, collagen and its degradation products, effectively providing the organism with nutrients. However, it is also possible for proteases to cause direct or indirect damage to the host tissues, and thus be classified as virulence factors (Portenier 2003). *E. faecalis* isolated from hospitalized patients and patients with endocarditis have been found to produce increased levels of gelatinase compared with community isolates (Coque *et al.* 1995). In relation to endodontic infection, gelatinase activity was expressed in 70% of the 33 endodontic enterococcal isolates tested (Sedgley *et al.* 2005). Expression of the gelatinase gene was associated with increased adhesion of *E. faecalis* to dentine *in vitro* (Hubble *et al.* 2003) and enhanced biofilm formation in microtiter plates (Kristich *et al.* 2004). Gelatinase activity may also play a role in the long-term survival of *E. faecalis* in obturated root canals, as evidenced by higher viable counts of gelatinase-producing compared to gelatinase-negative variants of the microbe in an *ex vivo* model (Sedgley 2007).

### 1.5.5.4 Cytolysin

This is a plasmid-encoded toxin produced by beta-hemolytic *E. faecalis* isolates. It lyses erythrocytes, polymorphonuclear leukocytes and macrophages, kills bacterial cells and may lead to reduced phagocytosis (Portenier 2003). Epidemiological investigations
partly support a role for cytolysin in disease occurrence. Ike et al. reported that approximately 60% of *E. faecalis* clinical isolates derived from various sources were haemolytic, compared to only 17% of *E. faecalis* isolates derived from the faecal specimens of healthy individuals (Ike et al. 1987).

1.5.5.5 **Extracellular superoxide**

Superoxide anion is a highly reactive oxygen radical involved in cell and tissue damage. Extracellular superoxide production has been reported to be a common trait in strains of *E. faecalis*, with 87 out of a total of 91 clinical and community isolates found to produce detectable extracellular superoxide anion levels (Huycke et al. 1996). Isolates associated with bacteremia or endocarditis produced significantly higher extracellular superoxide levels than those from the stool of healthy subjects (Huycke et al. 1996), indicating its potential role as a virulence factor. In relation to an endodontic infection, production of superoxide by persisting *E. faecalis* cells within the root canal system could translate into continuing tissue damage at the periapex and an absence of healing. An imbalance between oxygen radical production in periapical lesions and its elimination has been suggested to contribute to periapical damage and bone loss in chronic apical periodontitis (Marton et al. 1993).

1.5.5.6 **Pheromones**

Pheromones are chromosomally encoded, small hydrophobic peptides which have a signalling function between *E. faecalis* cells. The transfer frequency of certain plasmids in *E. faecalis* is increased several-fold by the action of pheromones (Kayaoglu and Ørstavik 2004). Briefly, the latter phenomenon occurs as follows. The recipient strain
secretes pheromones corresponding to the plasmid which it does not carry. In response, the donor strain produces aggregation substance that provides tight contact between the recipient and donor strain, facilitating the conjugative transfer of the replicated plasmid. Once a copy of the plasmid is acquired, the recipient shuts off the production of that pheromone, but continues to secrete pheromones specific for other plasmids that it does not carry. Antibiotic resistance and other virulence traits, such as cytolysin production, can be disseminated among strains of \textit{E. faecalis} via the pheromone system (Kayaoglu and Ørstavik 2004). Response to pheromones was identified in 48\% of endodontic enterococcal isolates, suggesting the potential of these cells to transfer plasmid DNA between strains.

1.5.5.7 Virulence factors and Pathogenicity

In conclusion, for a bacterium to be pathogenic, it must be able to adhere to, grow on, and invade the host. It must then survive host defence mechanisms, compete with other bacteria, and produce pathological changes. With the virulence factors described above, \textit{E. faecalis} appears to possess the requisites to establish an endodontic infection and maintain an inflammatory response potentially detrimental to the host (Kayaoglu and Ørstavik 2004). Which, if any of these factors play a role in the pathogenesis of periradicular diseases remains to be elucidated (Rôças \textit{et al.} 2004).

1.6 The viable but non-culturable (VBNC) state

It has been observed that after an extended period of starvation, the number of cultivable cells as determined by plate counts declines, while the total number of cells
present, as determined by a direct count method, remains unchanged (Bogosian et al. 1998). While one explanation is that the non-culturable cells are dead, an alternative explanation is that the non-culturable cells have entered a state in which they are still viable but cannot be cultured by standard microbiological techniques (Lleo et al. 1998; Lleo et al. 2001). The viable but non-culturable state is believed to constitute a survival strategy adopted by bacteria when exposed to environmental stress (Barer and Harwood 1999). The hostile environmental conditions that have been described as inducing the activation of the VBNC state include low nutrient concentrations, low or high temperatures, high salinity and extreme pH. When in the VBNC state, bacteria are no longer culturable on conventional growth media, but the cells display active metabolism, respiration, membrane integrity, and gene transcription (Lleo et al. 2001).

To date, there are no adequate techniques to prove the viability of cells in the VBNC physiological state (Portenier 2003). Recovery of culturable cells from a population of non-culturable cells would provide convincing support for the VBNC hypothesis. The appearance of large numbers of culturable cells after the addition of nutrients to populations of non-culturable cells has been reported to occur via a process termed resuscitation. However, such recovery studies can be confounded by the presence of small numbers of culturable cells, which can grow in response to the addition of nutrients and give the illusion of resuscitation. Bogosian et al. prepared samples that contained only non-culturable cells of various enteric bacteria, and subjected them to either a temperature shift or a nutrient addition in order to determine their resuscitation potential. They did not yield any culturable cells (Bogosian et al. 1998).
It has also been suggested that the presence of culturable cells is required for the resuscitation of non-culturable cells. This possibility was tested by using a mixed culture recovery method, which evaluated a mixture of easily distinguishable culturable and non-culturable cells to determine which group of cells responded to various resuscitation techniques. The results showed that the only culturable cells yielded from the mixtures were from the initial culturable cells, indicating that the culturable cells were not capable of resuscitating the non-culturable cells (Bogosian et al. 1998). The authors concluded that the non-culturable cells were dead, questioning the existence of the VBNC state for enterococcal bacteria.

In an attempt to develop methods capable of detecting non-culturable bacteria and establishing their viability, numerous researchers have used a modification of the conventional PCR method by using RNA as the amplification template (Lleo et al. 2000; Williams et al. 2006). This method is referred to as reverse transcription-PCR or RT-PCR. Messenger RNA is a short-lived molecule which serves as a marker of viability and active replication. Lleo et al. reported that E. faecalis conserves its viability in the VBNC state by limiting protein synthesis to a few key proteins, such as the penicillin binding protein (\textit{pbp5}). Thus RT-PCR amplification of the \textit{pbp5}-encoding regions of \textit{E. faecalis} mRNA should allow the detection of VBNC \textit{E. faecalis} and therefore, enable us to determine whether endodontic infections harbor the bacterium in this state (Lleo et al. 2000). Williams et al. detected \textit{E. faecalis} in 7 out of 87 intra-canal samples that were negative by cultivation, suggesting that even when undetected by cultivation, \textit{E. faecalis} remains viable and may return to an active, pathogenic state. However, every sample that was positive by the RNA-based RT-PCR assay was also positive by the DNA-based qualitative PCR assay, indicating that viable
bacteria may simply have been present in numbers small enough to be detected by the more sensitive molecular methods but not by cultivation (Williams et al. 2006). Some caution must therefore be exercised when evaluating recent studies that purport to show *E. faecalis* in the VBNC state and studies claiming to show cellular resuscitation (Portenier 2003).

1.7 The ‘biofilm’ concept

Although the concept of a microbial biofilm is not a new one, it has not been until recent years that its importance in human disease has been recognized. A biofilm can be defined as a microbial community characterized by cells that are attached to a substratum, encased in a matrix of extracellular polymeric substance, and exhibiting altered growth phenotypes (Donlan and Costerton 2002). A complex system of cell-to-cell communication underlies this process. Bacteria themselves account for a variable fraction of the total biofilm volume, typically 5-35%. The remainder of the volume is comprised of extracellular matrix (del Pozo and Patel 2007).

The diversity of biofilm-associated infections is rising, with estimates indicating their involvement in more than 65% of all bacterial infections (Lewis 2001). A number of human bacterial diseases produce lesions characterized by the presence of biofilm. Examples include infections of the oral soft tissues and teeth, as well as the middle ear, gastrointestinal and urogenital tract. Biofilms have been observed on invasive medical devices, such as indwelling catheters, cardiac implants, and tracheal and ventilator tubing (Costerton et al. 1999).
Biofilms grow comparatively slowly, and infections are often slow to produce overt symptoms. Sessile bacterial cells release antigens and stimulate the production of antibodies, but these are ineffective in killing biofilm bacteria, and can in fact cause immune complex damage to surrounding tissues (Costerton et al. 1999). Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defence mechanisms. Antibiotic therapy typically suppresses symptoms of infection by killing free-floating bacteria shed from the attached population, but fails to eradicate those cells that are still embedded in the biofilm. When antimicrobial chemotherapy stops, the biofilm can act as a nidus for the recurrence of infection (Stewart and Costerton 2001).

1.7.1 Biofilm formation as a survival strategy

A major advantage to its colonizing species is the protection afforded by the biofilm structure from competing microorganisms, from environmental factors such as host defence mechanisms, and from potentially toxic substances in the environment such as lethal chemicals and antibiotics (Socransky and Haffajee 2002). Biofilms also facilitate the processing and uptake of large, complex nutrient molecules that could not be efficiently degraded by an individual bacterium. The breakdown of these nutrients requires the sequential action of a range of complementary extracellular enzymatic profiles (Svensater 2004). Other benefits of a community microbial lifestyle are cross-feeding (one species providing nutrients for another), the removal of potentially harmful metabolic products, and the development of an appropriate physicochemical environment to facilitate microbial survival (Socransky and Haffajee 2002).
In many aspects the biofilm mode of growth is a survival strategy, and may therefore be favoured by bacteria colonizing the harsh environmental conditions of the root canal. This aspect is supported by the finding that clinically isolated *E. faecalis* species possess many of the characteristics of a biofilm style of growth, including increased adherence capacity, increased virulence factors and increased resistance to antimicrobials (George *et al.* 2005).

### 1.7.2 Evidence for biofilm structures in Endodontics

Currently, limited information is available on the development or the physiology of biofilms in the root canal (Figdor and Sundqvist 2007). An accurate depiction of the ultrastructural features of the biofilm of infected root canals was first reported by Nair, who observed ‘dense aggregates sticking to the canal walls’ and forming layers of bacterial condensations. Amorphous material filled the interbacterial spaces and was interpreted as an extracellular matrix of bacterial origin (Nair 1987).

Noiri *et al.* examined the surfaces of extracted teeth, root tips and extruded gutta-percha points acquired from teeth with refractory periapical periodontitis and noted bacterial biofilms in 9 of the 11 samples. The gutta-percha surface was almost completely covered with a glycocalyx-like structure, with filaments, long rods, and spirochete-shaped bacteria predominating. Planktonic cells were observed at a distance from the glycocalyx structure in some areas. The findings suggested that bacterial biofilms formed in extra-radicular areas were related to refractory periapical periodontitis (Noiri *et al.* 2002). A follow up *in vitro* investigation concluded that *E. faecalis, Strep.*
*sanguis*, *Strep. intermedius*, *Strep. pyogenes* and *Staph. aureus* produced single-species biofilms on the surfaces of gutta-percha points. High serum concentrations were required for the biofilm to establish (Takemura *et al.* 2004).

Distel *et al.* noted the ability of pure cultures of *E. faecalis* to form biofilm structures on the canal walls of both calcium hydroxide medicated and non-medicated root canals. Colonies of *E. faecalis* consisted of bacteria embedded in branching networks of filamentous material representing the extracellular polysaccharide matrix. The authors suggested that biofilm formation may be a mechanism that allows this organism to resist treatment (Distel *et al.* 2002).

Nair *et al.* assessed the intracanal microbial status of the apical root canal system of the mesial roots of human mandibular first molars with primary apical periodontitis. Periradicular surgery was performed on 16 teeth immediately after the completion of one-visit endodontic treatment. Fourteen of the sixteen mandibular molars examined showed residual infection of the mesial roots after instrumentation, irrigation with NaOCl, and obturation were completed. The infectious agents were mostly located in the uninstrumented recesses of the main canals, the isthmus communicating between them, and in accessory canals. They were mostly organized as biofilms (Nair *et al.* 2005).
1.7.3 Antimicrobial resistance of Biofilms

Susceptibility tests of *in vitro* biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture (Stewart and Costerton 2001). Abdullah *et al.* demonstrated that *E. faecalis* grown as a biofilm was more resistant to chlorhexidine and povidone iodine than the same strain grown in planktonic suspension (Abdullah *et al.* 2005).

It is likely that biofilms evade antimicrobial challenges by multiple mechanisms. One mechanism of biofilm resistance is the failure of an agent to penetrate the full depth of the biofilm. The biofilm matrix is known to retard the diffusion of some antibiotics and solutes in general (Costerton *et al.* 1999). The negatively charged polymers within the matrix may neutralize strong oxidizing agents, such as sodium hypochlorite, making it difficult for them to penetrate and kill microorganisms (Stewart *et al.* 2001).

The second mechanism involves the physiological state of biofilm microorganisms. Bacterial cells residing within a biofilm grow more slowly than planktonic cells, and consequently take up antimicrobial agents at a reduced rate. Furthermore, the depletion of nutrients can force bacteria into a dormant growth phase in which they are protected from killing (Portenier *et al.* 2005).

The third suggested mechanism responsible for antimicrobial tolerance is that microorganisms within the biofilm experience metabolic heterogeneity. The physical
conditions available to support bacterial growth, such as pH, ion concentration, nutrient availability, and oxygen supply, vary throughout the biofilm (Distel et al. 2002). Most antibiotics are not active in a variety of physical environments. Aminoglycosides for instance are more effective against bacteria growing in aerobic conditions than the same microorganism growing anaerobically (Costerton et al. 1999); therefore not all cells within the biofilm will be affected in the same way.

It is evident that oral microorganisms have the capacity to respond and adapt to changing environmental conditions (Bowden and Hamilton 1998). Individual microorganisms are able to sense and process the chemical information from the environment and adjust their phenotypic properties accordingly. Quorum sensing refers to the bacterial cell-to-cell communication mechanism for controlling cellular functions within biofilm structures. The signalling is mediated by diffusible molecules which, when present in sufficient concentrations, serve to modify gene expression in neighbouring microorganisms. Quorum sensing is known to be involved in the regulation of several microbial properties, including virulence and the ability to form biofilms, incorporate extracellular DNA and cope with environmental stress (Cvitkovitch et al. 2003).

The proximity of individual bacteria in biofilms increases the opportunity for gene transfer, making it possible to convert a previously avirulent organism into a highly virulent pathogen, or a bacterium that is susceptible to antimicrobials into a resistant one (Potera 1999). This potential for gene transfer within biofilms is particularly significant in the case of *E. faecalis*, because a number of its virulence factors are
encoded on transmissible plasmids. These include collagenase, gelatinase and adhesins, all having the potential to contribute to the survival of this species in the root canal (Distel et al. 2002).

A recent study demonstrated that over 80% of the bacteria isolated from acute endodontic abscesses or cellulitis had the ability to coaggregate with genetically distinct bacteria from the same site (Khemaleelakul et al. 2006). The significance of this is that coaggregated bacteria in biofilms might be able to transfer genetic material. Evidence for interspecies gene transfer was produced recently when it was shown that horizontal exchange of antibiotic resistance can occur between different bacterial species within root canals. Transfer of a conjugative plasmid carrying erythromycin resistance between 2 endodontic infection-associated species, S. gordonii and E. facealis in an ex vivo tooth model was observed (Sedgley et al. 2008).

Finally, it has been speculated that a sub-population of microorganisms exists within a biofilm which are known as ‘persisters’. These constitute a small percentage of the original population and are thought to represent a highly resistant phenotypic state that is resistant to killing by antimicrobial agents (Stewart and Costerton 2001). This hypothesis is supported by studies indicating that immature or newly formed biofilms are significantly less susceptible to antibiotics than corresponding planktonic cells, even though the formed layer is too thin to pose a barrier to penetration to either medication or metabolic substrates (Lima et al. 2001; Spratt et al. 2001).
1.8 Antimicrobial agents

Antimicrobial agents have generally been developed and optimized for their activity against fast growing, dispersed populations containing a single micro-organism. Unfortunately the effectiveness of such agents does not translate to the in vivo conditions present on the tooth surface, where bacteria grow as biofilms (Svensäter 2004). The biofilm structure may go some way to explain the frequent discrepancy observed in studies testing the antimicrobial effectiveness of endodontic irrigants and medicaments clinically, and in vitro.

1.8.1 Sodium Hypochlorite

Sodium hypochlorite is the most widely used irrigating solution. It has broad spectrum antimicrobial activity, rapidly killing vegetative and spore-forming bacteria, fungi, protozoa, and viruses (Siqueira et al. 2007). It exerts its antibacterial effect through its ability to oxidise and hydrolyse cell proteins and to some extent, osmotically draw fluids out of cells due to its hypertonicity (Pashley et al. 1985). When hypochlorite contacts tissue proteins, nitrogen, formaldehyde, and acetaldehyde are formed within a short time and peptide links are broken, resulting in protein dissolution. Sodium hypochlorite is thus a potent antimicrobial agent and additionally possesses the unique ability to dissolve necrotic tissue and organic components of the smear layer (Haapasalo 2005).

In vitro studies unequivocally support the strong antibacterial activity of sodium hypochlorite. Siqueira et al. reported that 4% NaOCl was significantly more effective
than saline solution in disinfecting root canals inoculated with *E. faecalis* (Siqueira et al. 1997). In an agar diffusion study, NaOCl at concentrations of 2.5% and 4% was effective against four black-pigmented obligately anaerobic bacteria and four facultative bacteria (Siqueira et al. 1998). Vianna et al. tested three gram-negative anaerobes typically isolated from primary apical periodontitis: *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia*. All three species were effectively killed within 15 seconds when exposed in a broth suspension to NaOCl at concentrations ranging from 0.5 to 5% (Vianna et al. 2004). Berber et al. assessed the efficacy of 0.5%, 2.5% and 5.25% NaOCl irrigation in human extracted teeth infected for 21 days with *E. faecalis*. The study protocol involved comparing various preparation techniques and either hand or rotary instrumentation. Following preparation, root canals were sampled and dentine chips obtained to assess dentine tubule disinfection. For all preparation techniques and at all depths of dentine tested, 5.25% NaOCl was shown to be the most effective irrigant solution tested, followed by 2.5% (Berber et al. 2006).

The antifungal efficacy of sodium hypochlorite was investigated by Waltimo et al. Both 5% and 0.5% NaOCl achieved effective killing of *Candida albicans* after a 30 second exposure. Sodium hypochlorite concentrations of 0.05% and 0.005% were too weak to kill the yeast, even after 24 hours of incubation (Waltimo et al. 1999). The antifungal properties of NaOCl were less impressive when Sen et al. used root sections of teeth inoculated with *C. albicans* and exposed them to 1% NaOCl, 5% NaOCl and 0.12% chlorhexidine for variable times. The smear layer had an inhibitive effect on the antifungal properties of the test agents. In its presence, antifungal activity was observed only in the 1 hour treatment groups for all solutions. When the smear layer was absent, 5% NaOCl was the first to display antifungal activity after 30 minutes (Sen et al. 1999).
Radcliffe et al. observed that E. faecalis was more resistant to the antimicrobial activity of hypochlorite compared to the yeast C. albicans (Radcliffe et al. 2004).

Byström and Sundqvist evaluated the effect of 0.5% NaOCl on fifteen single-rooted teeth in vivo. Each tooth was treated at five appointments, and the presence of bacteria in the root canal was studied on each occasion. No antibacterial intracanal dressings were used between appointments. When 0.5% NaOCl was used, no bacteria could be recovered from twelve of fifteen root canals at the fifth appointment, compared with eight of fifteen control root canals which had no recoverable bacteria after the use of saline irrigation. The results were statistically significant in favour of NaOCl, but the study highlighted that not all root canals could be rendered bacteria free through mechanical debridement and NaOCl irrigation, even after several appointments (Byström and Sundqvist 1983; Byström and Sundqvist 1985).

More recent clinical investigations have confirmed the ability of NaOCl to greatly reduce microbial counts within a treated root canal, and even render a proportion of root canals free of cultivable microorganisms. Yet, independent of the mechanical technique used for preparation, the size of the apical preparation or the concentration of NaOCl irrigation used, about half the root canals will continue to harbor cultivable bacteria (Shuping et al. 2000; McGurkin-Smith et al. 2005; Siqueira et al. 2007).

Peculiene et al. studied the effect of instrumentation and NaOCl irrigation in previously root-filled teeth with apical periodontitis. Retreatment was carried out under aseptic
conditions using rubber dam. The root fillings were removed with hand instruments, and without the use of chloroform. After the first microbiological sample was taken, the canals were cleaned and shaped with reamers and Hedstroem files to a size 40 or larger, using 2.5% NaOCl and 17% EDTA. A second microbiological sample was then taken. Bacteria were initially isolated in 33 out of 40 teeth, with *E. faecalis* present in 21 teeth, *C. albicans* in 6 teeth, gram-negative enteric rods in 3 teeth and other microbes in 17 teeth. Following instrumentation and irrigation no enteric gram-negative rods or yeasts were found in the second sample. In contrast, *E. faecalis* persisted in 6 root canals, and other microbes were found in 5 canals (Peciuliene *et al*. 2001). This finding corroborates the results of the *in vitro* studies, indicating the increased resistance of *E. faecalis* to NaOCl when compared with *C. albicans* and other gram-negative rods.

The root canal milieu is a complex mixture of a variety of organic and inorganic compounds. The relative importance of these compounds in the inactivation of root canal disinfectants has been evaluated in an *in vitro* model. Dentine powder was shown to exert an inhibitory effect on the antibacterial effectiveness of 1% sodium hypochlorite. When hypochlorite was pre-incubated with dentine for 24 hours, the killing of *E. faecalis* required 24 hours (Haapasalo *et al*. 2000).

Evaluations of antibacterial efficacy as a function of sodium hypochlorite concentration reveal conflicting results. *In vitro* studies, particularly agar diffusion tests, generally report reduced antibacterial effectiveness of NaOCl following dilution (Yesilsoy *et al*. 1995; Siqueira *et al*. 1998) Gomes *et al*. tested *in vitro* the effect of various concentrations against *E. faecalis* using cell culture plates. The microbe was killed in
less than 30 seconds by the 5.25% solution, while it took 10 and 30 minutes for complete killing of the bacteria by 2.5% and 0.5% solutions, respectively (Gomes et al. 2001).

In a more clinically applicable *ex vivo* model, Siqueira et al. evaluated the impact of hypochlorite concentration using extracted human teeth that were inoculated with *E. faecalis* prior to instrumentation and irrigation with either 1%, 2.5%, or 5.25% NaOCl. No significant difference was noted between the three NaOCl solutions tested (Siqueira et al. 2000). Numerous clinical studies have similarly found no significant differences in antibacterial efficiency between 0.5% and 5% NaOCl solutions (Cvek et al. 1976; Byström and Sundqvist 1985). This suggests clinical factors such as the regular exchange and large volumes of irrigant may maintain the antibacterial effectiveness of the NaOCl solution, compensating for a reduced concentration.

One of the fundamental properties of sodium hypochlorite is its ability to dissolve organic matter inside the root canal system. This dissolution is dependent of three factors: frequency of agitation, amount of organic matter in relation to the amount of irrigant and the surface area of the tissue to undergo dissolution (Mohammadi 2008). Okino et al. evaluated the tissue dissolving capacity of sodium hypochlorite and chlorhexidine by placing weighed bovine pulp fragments in contact with 20mls of NaOCl or chlorhexidine within a centrifuge, until total dissolution took place. Sodium hypochlorite was the only agent tested that effectively dissolved the organic tissue. The dissolution speed was affected by concentration, with 2.5% NaOCl dissolving pulpal tissue faster than 1% and 0.5% NaOCl (Okino et al. 2004). These findings were
confirmed by Naenni et al., who used standardized samples of necrotic tissue obtained from pig palates for this dissolution study (Naenni et al. 2004).

The main disadvantage of using sodium hypochlorite as an endodontic irrigant is that it is highly toxic at high concentrations, and tends to induce tissue irritation on contact (Hauman and Love 2003). Zhang et al. evaluated the cytotoxicity of four concentrations of NaOCl (5.25%, 2.63%, 1.31%, and 0.66%) on fibroblasts grown on cell culture plates. The results showed that toxicity of NaOCl was dose-dependent (Zhang et al. 2003). Barnhart et al. measured the cytotoxicity of several endodontic agents on cultured gingival fibroblasts, and showed that iodine potassium-iodide and calcium hydroxide were significantly less cytotoxic than NaOCl (Barnhart et al. 2005).

1.8.2 Calcium Hydroxide

The efficacy of calcium hydroxide in endodontic therapy stems mainly from its bactericidal effects, its ability to stimulate the formation of calcified tissue and its capacity to denature protein, aiding the dissolution of pulpal remnants. Cvek popularised the use of calcium hydroxide in the endodontic treatment of incompletely developed tooth roots, exploiting its ability to produce sterile necrosis and subsequent calcification (Cvek, 1976). Currently, calcium hydroxide is recognised as one of the most effective antimicrobial dressings available for the purposes of endodontic treatment (Siqueira and Lopes 1999). Its antimicrobial activity is due to the release and diffusion of hydroxyl ions, which produce a highly alkaline environment incompatible with microbial survival. Hydroxyl ions are indiscriminately reactive with a range of
biomolecules, exerting their lethal effects on bacterial cells by damaging their cytoplasmic membranes and denaturing proteins and DNA (Siqueira and Lopes 1999).

Byström et al. examined the anti-bacterial effects of different medicaments and found a 4-week application of calcium hydroxide to be more effective than camphorated paramonochlorophenol or camphorated phenol. Calcium hydroxide was capable of rendering 97% of the canals culture-negative; while this was achieved in only 66% of the canals treated with paramonochlorophenol or camphorated phenol (Byström et al. 1985). Sjögren et al. found that dressing canals with calcium hydroxide for 7 days was sufficient to reduce canal bacteria to a level that produced a negative culture (Sjögren et al. 1991). The effectiveness of calcium hydroxide as an anti-bacterial agent was further confirmed in the study by Shuping et al., in which the addition of calcium hydroxide medicament after chemomechanical preparation with rotary Ni-Ti instrumentation and NaOCl irrigation increased the number of culture-negative canals from 61.9% to 92.5% (Shuping et al. 2000). These reports contrast with findings of little difference in treatment outcome between single and multiple-visit treatment using a calcium hydroxide dressing (Trope et al. 1999; Weiger et al. 2000; Peters and Wesselinck 2002).

For calcium hydroxide to act effectively as an intracanal dressing, the hydroxyl ions must be able to diffuse through dentine and pulpal tissue remnants. Studies have demonstrated that hydroxyl ions derived from a calcium hydroxide medication do diffuse through root dentine, but the pH values decrease as a function of distance from the main canal. Tronstad et al. showed that the pH in the root canal was greater than 12.2, whereas in the most peripheral dentine, the pH ranged from 7.4 to 9.6 (Tronstad et
Similar results were reported by Nerwich et al., who also showed that there was a difference in the rate and amount of diffusion, with cervical dentine increasing in pH much more rapidly than apical dentine. This was attributed to an increased concentration and diameter of dentinal tubules in the cervical compared to the apical third of the root. It was also found that a time period of 1-7 days was required for the hydroxyl ions to reach the outer root dentine in this in vitro model, and 3-4 weeks was needed to reach peak pH levels peripherally (Nerwich et al. 1993). This has implications for establishing appropriate time-frames for dressing changes.

In order to have antibacterial effects within dentinal tubules, the ionic diffusion of calcium hydroxide needs to exceed the dentine’s buffering capacity, reaching pH levels sufficient to destroy bacteria (Siqueira and Lopes 1999). Haapasalo clearly showed that dentine powder abolished the killing of E. faecalis by calcium hydroxide. Without the dentine, the test organism was eliminated within minutes, whereas with the dentine powder added, no reduction in the bacterial colony-forming units could be measured, even after 24 hours of incubation (Haapasalo et al. 2000). The strong impact of dentine on the antibacterial action of a saturated calcium hydroxide solution has been attributed to the buffering action of dentine against alkali, with proton donors such as H$_2$PO$_4^-$, H$_2$CO$_3$ and HCO$_3^-$ found in the mineralized hydroxyapatite of dentine (Haapasalo et al. 2007). With a modification of the dentine powder model, Portenier et al. showed that organic material may also be capable of buffering calcium hydroxide because killing of E. faecalis by calcium hydroxide was completely prevented by 18% bovine serum albumin. This protein is used to simulate the high protein content of an inflammatory exudate which may be present within the root canal (Portenier et al. 2001). These findings must be treated with a degree of caution, however, as they are in vitro studies.
not necessarily representative of the clinical situation. In particular, the agents tested in these experiments are exposed to a much larger surface area of the dentine powder than would be the case clinically, a fact that is likely to have increased the overall buffering effect.

*In vitro* studies have demonstrated the capacity of calcium hydroxide to dissolve necrotic tissue, using porcine and bovine muscle tissue (Hasselgren *et al.* 1988; Turkun and Cengiz 1997). In addition, these studies found sodium hypochlorite and calcium hydroxide had an additive effect, with calcium hydroxide pre-treatment of root canals increasing the effectiveness of 0.5% NaOCl in achieving clean root canal walls as assessed by SEM (Turkun and Cengiz 1997). Calcium hydroxide also makes a significant contribution to the inactivation of lipopolysaccharides. Bacterial lipopolysaccharide (LPS) has a major role in the development of periapical bone resorption (Spångberg 2002). Treatment with an alkali such as calcium hydroxide affects the hydrolysis of the lipid moiety of bacterial LPS, resulting in the release of free hydroxyl fatty acids. Thus calcium hydroxide mediated degradation of LPS may be an additional important reason for the beneficial effects obtained with calcium hydroxide use in clinical endodontics (Spångberg 2002).

*E. faecalis* is well recognized for its capacity to resist the antibacterial effect of calcium hydroxide (Byström *et al.* 1985; Haapasalo and Ørstavik 1987; Ørstavik and Haapasalo 1990; Safavi *et al.* 1990; Siqueira and de Uzeda 1996). In fact its ability to withstand high pH levels is an identifying characteristic of this microorganism. Byström *et al.* showed that the pH needs to reach levels of 11.5 or higher to exert a bactericidal effect.
on *E. faecalis* (Byström *et al.* 1985). Investigations of the mechanisms involved in the resistance of *E. faecalis* to calcium hydroxide identified that its survival at high pH was related to the effective function of its proton pump. When cells were exposed to calcium hydroxide at pH 11.1 for 30 minutes, there was a 20-fold reduction in cell survival in the presence of the proton pump inhibitor CCCP (Evans *et al.* 2002). Furthermore, the buffering effects of dentine may not allow a sufficiently high pH to be achieved in the dentinal tubules (Haapasalo *et al.* 2000). The biofilm forming ability of *E. faecalis* constitutes another important survival strategy (Distel *et al.* 2002), while its proficiency at invading dentinal tubules affords the organism physical protection from chemo-mechanical root canal preparation and intracanal dressings (Love 2001). Haapasalo and Ørstavik reported that a calcium hydroxide paste failed to eliminate, even superficially, *E. faecalis* within the dentinal tubules (Haapasalo and Ørstavik 1987), a finding later supported by Heling *et al.* (Heling *et al.* 1992). Currently, investigations are focusing on identifying specific ‘stress’ genes that become upregulated following prolonged exposure to alkaline pH levels (Appelbe and Sedgley 2007).

Calcium hydroxide has great value in endodontics, but it is not a panacea. A limited antibacterial spectrum means that calcium hydroxide does not affect all members of the endodontic microbiota. The frequent isolation of enterococci in cases of endodontic failure has led some to question its routine use as an intracanal medicament, especially in re-treatment cases (Molander *et al.* 1998). In a study of retreatments, 11% of the root canals still contained bacteria after two consecutive dressings with calcium hydroxide. Two-thirds of these re-treatments failed (Sundqvist *et al.* 1998).
1.8.3 Chlorhexidine

This synthetic cationic bisbiquanide is highly efficacious against several gram-positive and gram-negative oral bacterial species, as well as yeasts. The positive charge of the chlorhexidine molecule interacts with the negatively charged phosphate group on microbial cell walls, thereby altering the cells’ osmotic equilibrium (Gomes et al. 2003). This increases the permeability of the cell wall, allowing the chlorhexidine molecule to penetrate into the bacteria. At low concentrations (0.2%) chlorhexidine exerts a bacteriostatic effect, by causing low molecular weight elements such as potassium and phosphorous to leak out of the cell. At higher concentrations (2%) chlorhexidine is bactericidal as precipitation of the cytoplasmic contents results in cell death (Gomes et al. 2003). Despite claims of biocompatibility, chlorhexidine is cytotoxic when in direct contact with human cells. A comparative study using fluorescence assays on human PDL cells showed similar cytotoxicity between 0.4% NaOCl and 0.1% chlorhexidine (Chang et al. 2001). Boyce et al. found chlorhexidine 0.05% to be uniformly toxic to both cultured human cells and microorganisms (Boyce et al. 1995).

Most comparative studies evaluating the effectiveness of chlorhexidine and sodium hypochlorite find their antibacterial effects in vitro and ex vivo to be similar at comparable concentrations. Oncag et al. evaluated the antibacterial properties of 5.25% sodium hypochlorite, 2% chlorhexidine and 2% chlorhexidine with 2.2% cetrimide. The root canals of extracted teeth were infected with E. faecalis and the antibacterial effects of the irrigants were examined after 5 minutes and 48 hours. Two percent chlorhexidine with cetrimide was significantly more effective against E. faecalis than 5.25% NaOCl at
5 minutes, although no significant differences could be observed when the same specimens were sampled at 48 hours (Oncag et al. 2003).

Two in vitro studies investigated the effectiveness of several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and three concentrations of chlorhexidine (0.2%, 1%, and 2%) in both liquid and gel forms, against various endodontic pathogens. All irrigants were effective in killing E. faecalis, but required vastly different times of exposure. Chlorhexidine in liquid form at all concentrations tested, and NaOCl at 5.25% were the most effective irrigants, eliminating the test organism within 30 seconds. The 0.2% chlorhexidine gel, on the other hand, required 2 hours contact time to produce a negative culture of E. faecalis (Gomes et al. 2001; Vianna et al. 2004).

Siqueira et al. tested the antibacterial effect of several endodontic irrigants against four black-pigmented gram-negative anaerobes and four facultative anaerobes by means of an agar diffusion test. The 4% and 2.5% concentrations of NaOCl were found to have a more pronounced antibacterial effect than 2% and 0.2% chlorhexidine. No significant difference was observed between the zones of inhibition recorded for the two concentrations of chlorhexidine (Siqueira et al. 1998).

Numerous studies use the agar diffusion method to compare the antibacterial efficacy of chlorhexidine, calcium hydroxide and their combination, usually against E. faecalis. The results consistently show chlorhexidine to be more effective than calcium hydroxide paste in eliminating the test organism, and generally chlorhexidine alone is
more successful than its combination with calcium hydroxide (Gomes et al. 2006; Ballal et al. 2007; Neelakantan et al. 2007; de Souza-Filho et al. 2008). The results of the agar diffusion method depend upon the molecular size, solubility and diffusion of the materials through the aqueous agar medium, the bacterial species and the number of bacteria inoculated, pH of the substrates in plates, agar viscosity, storage conditions of the agar plates, incubation time and the metabolic activity of the microorganisms (Gomes et al. 2006). Therefore, the inhibition zones may be more related to the materials’ solubility and diffusibility in agar than to their actual efficacy against the microorganisms. The antimicrobial activity of calcium hydroxide is related to its high pH, which causes the medicament to precipitate on agar, preventing its diffusion. This may explain the poor performance of calcium hydroxide when the agar diffusion method is used (Gomes et al. 2006).

A more clinically applicable model than the agar diffusion test uses extracted teeth which are instrumented, inoculated with a test organism, usually *E. faecalis*, and exposed to the antimicrobial agent. Investigations comparing the efficacy of chlorhexidine and calcium hydroxide at eliminating *E. faecalis* from infected dentinal tubules found chlorhexidine to be significantly more effective than calcium hydroxide paste or a mixture of the two (Schafer and Bossmann 2005; Ercan et al. 2006). Chlorhexidine achieves its optimal antimicrobial activity within a pH range of 5.5-7.0. It is likely that increasing the pH above 7.0 by adding calcium hydroxide will lead to the precipitation of chlorhexidine molecules and a reduction in its effectiveness (Mohammadi and Abbott 2009). Therefore the usefulness of mixing calcium hydroxide and chlorhexidine remains unclear and controversial (Athanassiadis et al. 2007).
As alluded to already, many in vitro studies use extracted bovine or human teeth mono-infected with *E. faecalis*. Chlorhexidine is known to be less effective against gram-negative bacteria than gram-positive taxa. Its efficacy against gram-positive taxa such as *E. faecalis* in laboratory experiments may therefore cause an over-estimation of its clinical usefulness (Zehnder 2006). This could at least partly explain why the potential benefits of chlorhexidine indicated by in vitro studies have not been fully realised in vivo. In a randomised clinical trial on the reduction of intracanal microbiota by either 2.5% NaOCl or 0.2% chlorhexidine irrigation, hypochlorite was found to be significantly more efficient than chlorhexidine in obtaining negative cultures (Ringel *et al.* 1982).

In another randomised clinical trial by Zamany *et al.*, a 2% chlorhexidine solution, used as a final irrigant, significantly decreased bacterial loads in root canals that had been irrigated with sodium hypochlorite during canal preparation. Cultivable bacteria were retrieved at the conclusion of the first visit in 1 out of 12 chlorhexidine-treated teeth, in contrast to the control group in which 7 out of 12 teeth showed growth (Zamany *et al.* 2003). A shortcoming of this otherwise well conducted study was that the final chlorhexidine rinse was compared to an identical procedure using sterile saline, thus not clarifying whether this regimen is superior to the use of hypochlorite as a final rinse.

When chlorhexidine is used as an irrigating solution, it has a relatively short effective exposure time in the root canal, which does not allow its full antibacterial action to be expressed. Chlorhexidine has the unique feature of imparting antibacterial substantivity to dentine. The positively charged molecules of chlorhexidine can adsorb onto dentine,
and prevent microbial colonisation for some time after medication. In order to achieve this substantivity, prolonged interaction is required to allow saturation of the dentine with chlorhexidine molecules. It has been suggested that this necessitates the placement of chlorhexidine as an intracanal medicament between appointments for at least seven days, rather than using it only as an irrigant (Athanassiadis et al. 2007).

Lin et al. placed a slow release device containing 5% chlorhexidine in cylindrical bovine root specimens infected with E. faecalis for seven days. Subsequently, no bacteria were recovered up to 500µm into the dentinal tubules. This compared to a significant reduction but a failure to eliminate viable bacteria when using 10ml of 0.2% chlorhexidine as an irrigant (Lin et al. 2003).

The antibacterial efficacy of 2% chlorhexidine gel, calcium hydroxide and a combination of both was assessed in teeth with chronic apical periodontitis. This randomised clinical trial by Manzur concluded that, when used as intracanal dressings, the antimicrobial efficacy of chlorhexidine gel and calcium hydroxide was comparable (Manzur et al. 2007). In the limited sample of thirty-three teeth, a one-week calcium hydroxide dressing reduced the bacteria-positive canals from 3 to 2, while medicating with 2% chlorhexidine gel showed a reversal of culture for one tooth, increasing the number of bacteria-positive canals from 4 to 5. Wang et al. evaluated the additional antibacterial effect of a 2 week intracanal dressing with a mixture of 2% chlorhexidine gel and calcium hydroxide. No significant difference could be detected in the percentage of positive samples before and after the placement of the medicament,
suggesting that the intracanal dressing did not significantly improve bacterial reduction (Wang et al. 2007).

Paquette et al. evaluated the antimicrobial effectiveness of 2% chlorhexidine gluconate liquid applied in vivo as an intracanal medicament for 7-15 days. No increase in the proportion of teeth with negative cultures or reduction in bacterial counts beyond that achieved after chemo-mechanical preparation could be found (Paquette et al. 2007). In fact, a significant increase in bacteria positive samples was reported, from 32% before to 54% after medication with chlorhexidine liquid. The use of a liquid form of chlorhexidine was suggested as a possible reason for its poorer-than-expected performance, with the authors indicating that its consistency may not be well suited for use as an intracanal medicament.

In another in vivo study, root canals that yielded positive cultures a week after complete chemo-mechanical preparation and camphorated paramonochlorophenol dressing were medicated with either calcium hydroxide, chlorhexidine or camphorated paramonochlorophenol for one week. There were no statistically significant differences in the percentage of negative cultures obtained with each medicament (Barbosa et al. 1997).

One of the major disadvantages of chlorhexidine is that it has no tissue solvent activity. It was proposed that this shortcoming could be compensated for by producing a gel formulation which increased its mechanical removal. This hypothesis has not been
validated by the literature. Okino et al. evaluated the tissue dissolving ability of 0.5%, 1% and 2.5% sodium hypochlorite, 2% chlorhexidine digluconate liquid, 2% chlorhexidine digluconate gel and distilled water. Distilled water and both solutions of chlorhexidine did not dissolve the pulp tissue within 6 hours (Okino et al. 2004). Unlike calcium hydroxide, chlorhexidine does not inactivate bacterial LPS (Tanomaru et al. 2003).

The prolonged antibacterial activity of chlorhexidine resulting from its substantivity, has been the focus of numerous investigations. Khademi et al. found that a 5 minute application of 2% chlorhexidine solution in a bovine root dentine model, induced substantivity for up to 4 weeks (Khademi et al. 2006). Rosenthal et al., using bovine root blocks, measured the substantivity of chlorhexidine in dentine after root filling for up to 3 months. After instrumentation and irrigation with NaOCl and EDTA, the test canals were irrigated with 2% chlorhexidine for 10 minutes before root filling. No chlorhexidine was used in the control group. Residual chlorhexidine activity was measured within the dentine at various time periods after filling, and antibacterial activity was clearly present even 3 months post-obturation (Rosenthal et al. 2004).

Another potential weakness of chlorhexidine is its susceptibility to the presence of organic matter. Haapasalo et al. showed the effect of 0.05% chlorhexidine against E. faecalis is reduced, but not totally eliminated, by the presence of dentine (Haapasalo et al. 2000), while no inhibition could be measured when a 0.5% solution was used. The strongest inhibitor of chlorhexidine was bovine serum albumin, with more than 10% of E. faecalis cells still viable after 24 hours of incubation with the medicament (Portenier
et al. 2001). Inflammatory exudate, rich in proteins such as albumin, may thus weaken the antibacterial effects of chlorhexidine more so than the dentine will (Haapasalo 2005).

A suggested clinical protocol by Zehnder consisted of irrigation with sodium hypochlorite to dissolve the organic components, irrigation with EDTA to eliminate the smear layer and irrigation with chlorhexidine to increase the anti-microbial spectrum of activity and impart substantivity (Zehnder 2006). Although such a combination of irrigants may enhance the overall antimicrobial effectiveness, the possible chemical interactions of the irrigants must be considered (Mohammadi and Abbott 2009). The combination of chlorhexidine and sodium hypochlorite has been shown to result in the formation of a precipitate which has been found to occlude dentinal tubules (Basrani et al. 2007; Bui et al. 2008). This may interfere with the seal of the root filling and the combination should therefore be avoided.

1.8.4 Antibiotics

In light of the limitations of calcium hydroxide and chlorhexidine as intracanal medicaments, preparations containing antibiotics represent another approach to treating persisting endodontic infection.

The two most common antibiotic-containing commercial preparations currently available are Ledermix paste and Septomixine Forte. Septomixine Forte contains two
antibiotics – neomycin and polymyxin B sulphate. Neither of these can be considered suitable for use against the commonly reported endodontic bacteria, because of their inappropriate spectra of activity (Abbott et al. 1990). In addition, although the anti-inflammatory agent in Septomixine Forte, dexamethasone, is clinically effective, the triamcinolone component of Ledermix paste is considered to have less systemic side effects (Abbott et al. 1990).

1.8.4.1 Ledermix

Ledermix paste was developed by Schroeder and Triadan in 1960, primarily as a corticosteroid vehicle aimed at controlling pain and inflammation in vital pulp therapy. The antimicrobial properties were initially catered for by a formalin-based paste, while the inclusion of an antibiotic component was to compensate for what was perceived to be a possible corticoid-induced reduction in the host immune response (Schroeder 1981). Today, Ledermix paste consists of a combination of the tetracycline antibiotic demeclocycline HCl, at a concentration of 3.2%, and a corticosteroid, triamcinolone acetonide at a concentration of 1%, in a polyethylene glycol base (Athanassiadis et al. 2007).

Heling and Pecht evaluated the efficacy of Ledermix paste in the disinfection of dentinal tubules. Ledermix was effective in reducing the number of viable Staphylococcus aureus bacteria in dentinal tubules after 7 days of incubation and after recontamination (Heling and Pecht 1991). However, its antibacterial effect is limited to a small spectrum of bacteria. Lin et al. found that exposure to Ledermix for 7 days had no significant effect on the amount of viable Streptococcus sanguis bacteria in bovine
dentinal tubules (Lin et al., 2003). A further limitation of Ledermix paste is that increasing numbers of bacterial species have become resistant to tetracycline (Goodson and Tanner 1992). For instance, numerous in vitro antimicrobial susceptibility studies have revealed a significant proportion of enterococcal isolates to be resistant to tetracyclines (Dahlén et al. 2000; Geijersstam et al. 2006). Dahlén et al. found 4 out of 29 strains to be highly resistant to tetracycline, while Geijersstam et al. identified 17 tetracycline resistant isolates out of 59 (Geijersstam et al. 2006). The latter study observed antibiotic susceptibility patterns of E. faecalis isolated from endodontic infections in Finland and Lithuania to be similar, despite the differing antibiotic usage in these countries (Geijersstam et al. 2006).

The concentration of demeclocycline achieved within the dentinal tubules and periapical tissues varies as a function of time and location – the further from the canal, the lower the concentration achieved and this concentration decreases with time (Abbott et al. 1990). Abbott et al. showed dentinal tubules are the major supply route of the active components to the periradicular tissues, while the apical foramen is not as effective as a supply route (Abbott et al. 1988). Demeclocycline within the root canal itself is present in sufficiently high concentrations to be effective against susceptible species of bacteria. In the periradicular tissues and peripheral parts of the dentine, however, the concentration achieved through diffusion is insufficient to inactivate bacteria (Abbott et al. 1990).

Chu et al. conducted an in vivo study comparing the efficacy of disinfection of Ledermix, Septomixine and Calasept - a calcium hydroxide paste. Each tooth was
prepared using hand instrumentation and repeatedly irrigated with 0.5% sodium hypochlorite. Each medicament was spiralled into the root canal and left for seven days. Bacteriological samples were taken before and after the two-visit endodontic treatment. After seven days, the percentages of canals with positive growth after dressing with Ledermix, Septomixine or Calasept, were 48% (13 of 27), 31% (8 of 26) and 31% (11 of 35), respectively. These differences were not statistically significant (Chu et al. 2006). There was a marked similarity in microbiological outcomes achieved despite the use of different inter-appointment dressings.

Trope et al. evaluated the relationship of intracanal medicaments to endodontic flare ups. Formocresol, Ledermix, and calcium hydroxide were used to dress 474 teeth in strict sequence irrespective of the presence or absence of symptoms or radiographic signs of apical periodontitis. No significant difference in the flare-up rate among the three intracanal medicaments was detected (Trope 1990). Ehrmann et al. showed that greater postoperative pain relief was achieved when teeth were medicated with Ledermix paste compared to calcium hydroxide (Ehrmann et al. 2003). Dentine acts as a slow release mechanism for triamcinolone to the periodontal tissues, which is the probable basis of its long-acting therapeutic effect (Schroeder 1981).

One of the drawbacks of using Ledermix as an intracanal medicament is its potential to cause tooth discolouration. It is well known that uptake of tetracyclines into hard tissues can produce colour changes. Tetracycline uptake was thought to be limited to periods of active calcification (Lambrou et al. 1977), but it is now recognized that even fully mineralized teeth can undergo colour changes resulting from topical exposure to
tetracycline (Bjorvatn et al. 1985). Kim et al. found that after 12 weeks, teeth dressed with Ledermix paste and exposed to sunlight, acquired a dark grey-brown staining. This did not occur when the teeth were kept in the dark. More severe staining was noted when Ledermix paste filled the pulp chamber than when the paste was restricted to below the cemento-enamel junction (Kim et al. 2000). In a similar study, immature teeth became more stained than their mature counterparts undergoing identical treatment. This was attributed to a greater number and wider diameter of dentinal tubules in immature teeth, allowing greater diffusion to take place (Kim et al. 2000).

In addition to its antimicrobial properties, Ledermix paste has important applications in the prevention and treatment of inflammatory root resorption following dental trauma. Triamcinolone is a highly active steroid, known to suppress inflammation and have an inhibitory effect on osteoclastic activity (Neuberger 1975). It was demonstrated to have a direct inhibitory effect on dentinoclasts (Pierce et al. 1988). Tetracycline has been shown to possess antimicrobial activity and have antiresorptive properties (Chang et al. 1994; Keller and Carano 1995). Abbott et al. demonstrated that both the antibiotic and corticosteroid components of Ledermix paste penetrate the dentinal tubules and the cementum and are readily available at the root surface. The rate of diffusion is significantly greater when the cementum is absent from the root surface, as may be the case in the root surfaces of traumatised teeth (Abbott et al. 1988).

Pierce and Lindskog reported that root canal treatment with intracanal Ledermix introduced 3 weeks after 1-hour delayed replantation of extracted teeth was effective against inflammatory root resorption (Pierce and Lindskog 1987). More recent studies
have evaluated the effect of immediate placement of intracanal Ledermix on root resorption of delayed replanted teeth in monkey and canine models. Immediate placement of intracanal Ledermix paste in replanted monkey and dog teeth resulted in more complete healing and less unfavourable healing including inflammatory root resorption and replacement resorption compared to root-filled replanted teeth (Wong and Sae-Lim 2002; Chen et al. 2008). When the individual influence of corticosteroid and tetracycline on external root resorption was assessed, there was no statistically significant difference between the Ledermix group and the triamcinolone group in the degree of favourable healing or the remaining root mass, while the tetracycline group showed less favourable healing and reduced root mass (Chen et al. 2008). The steroid component therefore appears largely responsible for the anti-resorptive effect of Ledermix paste, although the antimicrobial contribution of tetracycline may play an important adjunctive role. Ledermix paste treated canine roots had significantly more healing and less resorption than roots treated with calcium hydroxide, and consequently maintained significantly more root mass (Bryson et al. 2002). These observations suggest that Ledermix is highly effective in treating resorption and promoting favourable periodontal healing in delayed replantation cases.

In conclusion, the range of activity of demeclocycline in Ledermix, and the concentrations of this antibiotic established within the root canal system are not sufficient to achieve predictable antibiosis for an adequate length of time. Nevertheless, Ledermix is an effective intracanal medicament for the control of postoperative pain associated with acute apical periodontitis (Ehrmann et al. 2003) and vital pulp therapy (Negm 2001). It also has important applications in the prevention and treatment of inflammatory root resorption (Pierce and Lindskog 1987). These benefits need to be
balanced with the clinical concern of possible discolouration as a result of its use (Kim et al. 2000; Kim et al. 2000).

**1.8.4.2 Odontopaste**

Odontopaste was released in February 2008 by Australian Dental Manufacturing. It contains the antibiotic clindamycin hydrochloride 5% and triamcinolone acetonide 1% and now also comes premixed with 1-2% calcium hydroxide. The manufacturers of Odontopaste claim that this formulation will not cause dentinal discolouration as seen with Ledermix. At this stage there is no published data available to validate Odontopaste as an effective intracanal medicament.

The key antimicrobial component of Odontopaste is clindamycin, which has proven effectiveness against a comprehensive spectrum of microbes associated with endodontic infections, including *Actinomyces, Eubacterium, Fusobacterium, Propionibacterium*, microaerophilic *streptococci, Peptococcus, Peptostreptococcus, Veillonella, Prevotella*, and *Porphyromonas* (Pharmacists 1998). Only a small number of reports, however, describe the use of clindamycin as an intracanal medicament.

Gilad et al. found clindamycin to be an effective antimicrobial agent in extracted human teeth. In an initial experiment using a pure culture of *Prevotella intermedia*, all seven experimental teeth treated with clindamycin fibers demonstrated complete elimination of this organism when growth was assessed by either paper point or crushed tooth sampling. When the effect of clindamycin fibers on a mixed inoculum was assessed, a
significant reduction in *Fusobacterium nucleatum* and *Prevotella intermedia* was noted. In contrast, the clindamycin fibers failed to significantly reduce the levels of *Peptostreptococcus micros*. Total bacterial elimination failed to be achieved in any of the specimens inoculated with a mixed culture made up of *F. nucleatum*, *P. micros* and *P. intermedia* (Gilad et al. 1999).

Lin *et al.* sought to evaluate and compare the antibacterial effect of clindamycin and tetracycline in bovine dentinal tubules infected with *Streptococcus sanguis*. Following a 1 week exposure, the amount of viable bacteria in the dentinal tubules was significantly reduced through the use of clindamycin, while tetracycline yielded results similar to those of controls. Neither tetracycline nor clindamycin rendered any dentine samples bacteria-free (Lin *et al.* 2003).

Molander *et al.* evaluated the effects of clindamycin when used as an intracanal dressing on 25 teeth with necrotic pulps and periapical radiolucencies. A 150 mg clindamycin capsule was mixed with sterile saline to a paste consistency and placed into the infected root canals. The presence or absence of bacteria was determined in samples taken immediately after removal of the dressing and after a period of 7 days, during which the canals were filled with sampling fluid. Bacteria were recovered from 4 and 6 teeth respectively. Enterococci constituted the dominant flora in the teeth with persisting infection. Although there was an absence of direct comparison, these results indicated that clindamycin was not superior to calcium hydroxide in the eradication of bacteria from the root canal and its use was therefore not recommended (Molander *et al.* 1990).
*E. faecalis* generally displays intrinsic resistance to clindamycin (Singh *et al.* 2002) although Geijersstam *et al.* noted that 2 of the 59 endodontic isolates they tested did not display the classic LS\(_A\) phenotype which confers intrinsic resistance to streptogramin A and lincosamides such as clindamycin. These aberrant isolates were thus unusually susceptible to clindamycin and a further 3 isolates had reduced resistance to the antibiotic (Geijersstam *et al.* 2006). Similarly, Dahlén *et al.* found 25 out of the 26 *E. faecalis* species isolated from root canals to be resistant to clindamycin (Dahlén *et al.* 2000).

**1.8.4.3 Combinations of antibiotics and calcium hydroxide**

In order to compensate for its limited antibacterial efficacy and produce a dressing that has both antimicrobial and anti-inflammatory activity, Ledermix paste can be combined with calcium hydroxide in a 50:50 mixture. *In vitro* studies investigating this combination have generated contradictory results. Taylor *et al.* found that combining the two preparations marginally increased the anti-bacterial activity of Ledermix paste against the microorganisms *L. Casei* and *S. mutans* (Taylor *et al.* 1989). Seow, however, showed that for *S. sanguis* and *S. aureus*, the addition of only 25% of calcium hydroxide to Ledermix, converted the original zone of complete inhibition to one of only partial inhibition, thus countering claims of any synergistic activity of this mixture (Seow 1990). It has been shown that the 50:50 mixture results in slower release and diffusion of the active components of Ledermix paste (Abbott *et al.* 1989). Abbott hypothesized that an equal parts mixture of Ledermix and calcium hydroxide paste is likely to have more potent and longer lasting effects than when Ledermix is used alone, and less frequent changing of the dressings will be necessary (Abbott *et al.* 1989).
Molander and Dahlén evaluated the intracanal antibacterial potential of two antibiotic/calcium hydroxide mixtures against enterococci in vivo. Fifty-five teeth in which enterococci were present were dressed for one month with either tetracycline or erythromycin, each mixed with calcium hydroxide. The tetracycline mixture was effective against enterococci in 22 teeth (79%). In 7 teeth, other microorganisms were recovered, resulting in a total antimicrobial effect of 54%. The overall antimicrobial effect of this combination was deemed to be relatively weak (Molander and Dahlén 2003). The corresponding results for erythromycin were 96% and 56% respectively. Limitations of this study include the 3 week interval between the pre-intervention microbiological sampling of the canals to determine the presence of enterococci and the experimental dressing placement. During this time the teeth were dressed with calcium hydroxide. We are thus unable to conclude whether the treatment outcome was the result of the antibiotic mixture or the effects of calcium hydroxide alone. The high prevalence of microorganisms detected in the second sample which were absent in the initial sampling suggests the high likelihood of contamination, possibly due to ineffective temporization. Möller highlighted the specific concern of bacterial contamination in endodontic microbial studies, and identified certain microbial species which should be suspected to be contaminants – these include *Staphylococcus epidermidis* and lactobacilli (Möller 1966), which were identified on numerous occasions in the post-treatment samples in this study.

1.9 Susceptibility of biofilms to antimicrobials

Spratt et al. used a simple biofilm model to evaluate the effectiveness of a range of commonly recommended antimicrobial irrigants against monocultures of five root canal
isolates: *Prevotella intermedia*, *Peptostreptococcus micros*, *Streptococcus intermedius*, *Fusobacterium nucleatum*, and *Enterococcus faecalis*. Single species biofilms were generated on membrane filter discs and incubated with colloidal silver, 2.25% sodium hypochlorite, 0.2% chlorhexidine, 10% iodine or the phosphate buffered saline control. The effectiveness of an agent was dependent on the organism in the biofilm, and on the contact time. Iodine and NaOCl were more effective than chlorhexidine except against *P. micros* and *P. intermedia* where they were all 100% effective. Iodine and NaOCl elicited a 100% kill after 1 h incubation for all strains used. None of the agents were effective against *F. nucleatum* after 15 min but NaOCl, iodine and chlorhexidine were all effective after 1 h. Colloidal silver was generally ineffective. Sodium hypochlorite was the most effective antimicrobial overall, successfully achieving 100% killing of *E. faecalis* at both 15 and 60 minute time intervals. Iodine was only 100% effective after 60 minutes of exposure. Although there was clear evidence of a reduction in bacterial counts after 15 minutes with both chlorhexidine and iodine, both agents left in excess of $10^7$ colony forming units (Spratt *et al.* 2001). The results of this *in vitro* study are limited by the absence of dentine in the experimental model, which is known to have an inhibitive effect on antibacterial agents (Haapasalo *et al.* 2000). In addition, there is no accounting for the complexities of root canal anatomy nor the polymicrobial nature of root canal infection, both factors that may complicate disinfection.

The susceptibility of *E. faecalis* biofilms to antibiotic and chlorhexidine-based medicaments was evaluated by Lima *et al.* (Lima *et al.* 2001). One and three day biofilms were grown on cellulose nitrate membranes and exposed to the test agents for 24 hours. The formulations containing 2% clindamycin as the active antimicrobial did not achieve any reduction in the bacterial biofilm, with one formulation actually
generating bacterial overgrowth. The association of clindamycin with metronidazole significantly reduced the number of cells in 1-day biofilm compared to the control. Chlorhexidine-containing formulations were able to render a portion of the membranes bacteria-free, and were more effective than the antibiotic formulations tested (Lima et al. 2001).

Abdullah et al. investigated the variable efficacy of selected endodontic antimicrobials on a clinical isolate of *E. faecalis* grown as either a biofilm on cellulose nitrate membrane filters or as a planktonic suspension. Each bacterial presentation was exposed to calcium hydroxide, 0.2% chlorhexidine, EDTA, 10% povidone iodine or 3% sodium hypochlorite. Sodium hypochlorite was very effective against *E. faecalis* regardless of the growth conditions, achieving 100% killing after 2 minutes contact time. Calcium hydroxide was the only other agent equally effective against bacteria in a planktonic and biofilm presentation. While it could not achieve 100% kill, a greater than 4 minute exposure resulted in a bacterial reduction of 99.9%. Both 0.2% chlorhexidine and 10% povidone iodine were significantly less effective against the biofilm phenotype. The authors concluded that due to their capacity to dissolve organic tissue, sodium hypochlorite and calcium hydroxide were less inhibited in their antimicrobial activity by the exopolysaccharide matrix of the biofilm (Abdullah et al. 2005).

A 7 day polymicrobial biofilm was developed on the hemisections of root apices by Clegg et al. Each biofilm was separately immersed in 6% NaOCl, 3% NaOCl, 1% NaOCl, 2% chlorhexidine, 1% NaOCl followed by BioPure MTAD or sterile phosphate
buffered saline (PBS) for 15 minutes. Only 6% NaOCl was capable of both rendering bacteria nonviable and physically removing the biofilm. One percent and three percent NaOCl showed disruption and physical removal of bacteria when viewed under the SEM; however, both gave rise to bacterial colonies when dentinal shavings were cultured, indicating that bacteria had escaped the effects of the irrigants, probably by invading the dentinal tubules. No cultivable organisms were found after dentine sections were placed in 2% chlorhexidine, but when the samples were viewed under a scanning electron microscope, the biofilm was virtually intact. This was attributed by the authors to potential fixation of cells by chlorhexidine (Clegg et al. 2006).

The superiority of NaOCl was also reported by Dunavant et al., who found that both 6% and 1% NaOCl were efficient in eliminating *E. faecalis*, achieving >99.99% and 99.78% biofilm elimination, respectively. In comparison, 2% chlorhexidine achieved only a 60.5% bacterial reduction after a 5 minute exposure. This study used a novel *in vitro* testing system, in which a 24-hour biofilm was established on ceramic coupons in a flow cell system (Dunavant et al. 2006). Fluid flow is considered a principal determinant of the biofilm structure (Purevdorj et al. 2002). Smooth, dense and stable biofilms are formed at relatively high shear stress (Liu and Tay 2001). In addition to influencing structure, hydrodynamic conditions also influence biofilm density and strength, which in turn influences the diffusion of nutrients and signals through the biofilm (Purevdorj et al. 2002) and affects the dispersal of cells from the biofilm surface (Stoodley et al. 2002). The fluid flow in a root canal system is generated by the inflammatory exudate that forms subsequent to pulpal necrosis. Studies have demonstrated that occlusal forces can induce retrograde fluid movement into the apical portion of the root canal (Kishen 2005). This cyclic influx of tissue fluid into the apical
portion of the root canal may promote the persistence of bacterial biofilms in this region by providing a source of nutrients and exerting a shear force on the biofilm structure.

Single-species biofilms of *E. faecalis*, *S. aureus*, *C. albicans*, *P. intermedia*, *P. gingivalis*, *P. endodontalis* and *F. nucleatum* were used to test the antimicrobial activity of sodium hypochlorite and chlorhexidine (Sena *et al.* 2006). Ten day old biofilms were generated on cellulose nitrate membranes and immersed in either sodium hypochlorite or chlorhexidine for variable periods, with or without mechanical agitation. *P. intermedia*, *P. gingivalis*, *P. endodontalis* and *F. nucleatum* were eliminated in 30 seconds by all antimicrobial agents, while *S. aureus* and *E. faecalis* were the most resistant. Under conditions of mechanical agitation, *E. faecalis* was eliminated in 30 seconds by exposure to 5.25% sodium hypochlorite, 2.5% sodium hypochlorite or 2% chlorhexidine liquid, while 2% chlorhexidine gel needed 5 minutes to achieve microbial elimination. When the irrigants were not mechanically agitated, the effectiveness of the antimicrobial agents was reduced. *E. faecalis* was only eliminated after a 20 minute exposure to 2% chlorhexidine gel, while 2.5% NaOCl did not achieve total disinfection within the time limits of the experiment (60 minutes). It was hypothesized by the authors that the agitation improved the contact between the antimicrobial agent and the test organism in the biofilm, resulting in more efficient microbial elimination (Sena *et al.* 2006).

Williamson *et al.* subjected a 48-hour *E. faecalis* biofilm established on a sterile glass microscope slide to either 6% NaOCl or 2% chlorhexidine, in formulations with or without surface modifiers (Chlor-XTRA and CHX-Plus). The time of exposure was
either 1, 3 or 5 minutes. It was found that NaOCl with or without surface modifiers reduced bacterial numbers within the biofilm significantly better than chlorhexidine, although none achieved total bacterial elimination within the experimental time of exposure (Williamson et al. 2009).

Mixed-species biofilms of *F. nucleatum* and *P. micros* showed time-dependent synergy in growth and resistance to NaOCl. As the age of the biofilms increased, so did their resistance to NaOCl. Exposure of the 24-hour mixed-species biofilm to 0.005% NaOCl resulted in no recoverable bacteria, whereas this concentration of NaOCl had no significant effect on the viability of the 96-hour biofilm (Ozok et al. 2007). This finding is in accordance with earlier reports suggesting that as the age of the biofilm increases, its susceptibility to antimicrobials decreases (Stewart et al. 2001). The increased resistance is at least partly the result of increased numbers of bacterial cells and more exopolymeric substance. The extremely reactive NaOCl is chemically consumed on its way into the deeper layers of the biofilm, reducing its effectiveness (Stewart et al. 2001).

Chai et al. investigated the antimicrobial efficacy of 6 groups of antibiotics and calcium hydroxide against a two-day *E. faecalis* biofilm established on membrane filter discs. After a one hour exposure, the antimicrobial activity was neutralized by washing each disc five times in PBS, and then the colony forming units of the remaining viable bacteria on each disc were counted. It was concluded that erythromycin, oxytetracycline and calcium hydroxide were 100% effective in eliminating *E. faecalis* biofilm, whereas
ampicillin, co-trimoxazole, vancomycin and vancomycin followed by gentamicin were ineffective (Chai et al. 2007).

A recent study observed the qualitative effects of various antibiotics on endodontic bacteria cultured from necrotic pulps and grown as biofilms on dentine slices. The biofilms were grown for a period of 12 days, and exposed to 5 antibiotics (amoxicillin, clindamycin, metronidazole, doxycycline and azithromycin) at their respective minimum inhibitory concentrations. Scanning electron microscope images of biofilms exposed to each antibiotic for 3 and 8 days showed that none of the antibiotics had any observable effects on the biofilms (Norrington et al. 2008). As alluded to earlier, cells growing in biofilms have been shown to be up to 1000 times more resistant to antimicrobial agents than their planktonically growing counterparts (Ceri et al. 1999), so the ineffectiveness of antibiotics used at minimum inhibitory concentrations (MIC) is not surprising. This was also highlighted by Rossi-Fedele and Roberts who identified the MIC of plate-grown *E. faecalis* to tetracycline was 32 μg ml\(^{-1}\). In the root canal model, where the microbe had presumably established a biofilm, cells survived irrigation with a tetracycline concentration of 30 mg ml\(^{-1}\), almost 1000 times more than the concentration effective in the plate culture (Rossi-Fedele and Roberts 2007).

1.10 Rationale for experiment

Biofilm studies at this time constitute a considerably simplified microenvironment to that of the root canal system, in which complex anatomy, inhibitive actions of dentine and organic matter and a polymicrobial infection exponentially complicate the
disinfective process. Interestingly, in the field of endodontics, the evidence obtained from biofilm research appears more consistent with the findings of clinical research, compared to bench top studies. This is particularly so in relation to chlorhexidine, which has gained popularity as a result of its excellent performance in vitro, (Gomes et al. 2006; Ballal et al. 2007; Neelakantan et al. 2007; Filho et al. 2008) yet failed to fully realize its promise in vivo (Ringel et al. 1982). Perhaps this is an indication of the importance of the biofilms in the endodontic milieu and highlights the validity of using the biofilm model when testing the efficacy of endodontic disinfection agents in vitro.

The most commonly used endodontic medicaments in Australia are calcium hydroxide and the antibiotic/steroid combination of Ledermix paste (Heithersay 1984). Recently a new product, Odontopaste, has entered the market and been promoted as a non-staining alternative to Ledermix. In practice, many dentists and endodontists empirically combine Ledermix and calcium hydroxide in a 50:50 mixture. Presumably, similar practices will be adopted by practitioners who substitute Ledermix with Odontopaste. No research has been conducted on the antimicrobial efficacy of Ledermix or Odontopaste in the context of a microbial biofilm. Combinations of these medicaments with calcium hydroxide are also yet to be tested in a biofilm model, while calcium hydroxide on its own has only been evaluated in a handful of biofilm studies (Abdullah et al. 2005; Chai et al. 2007).

An in vitro study was thus devised to test the antimicrobial efficacy of some commonly used endodontic medicaments. E. faecalis was selected as the test microorganism because of its apparent association with persistent disease (Molander et al. 1998;
Sundqvist et al. 1998; Pinheiro et al. 2003; Siqueira and Rôças 2004; Gomes et al. 2008), and as a means of comparison to other single species biofilm studies (Lima et al. 2001; Spratt et al. 2001; Abdullah et al. 2005; Dunavant et al. 2006; Chai et al. 2007; Williamson et al. 2009). A dense biofilm was generated to evenly cover the substrate surface. A mature biofilm represents a highly organized structure which is less susceptible to antimicrobial effects (Stewart et al. 2001), and may therefore present a greater and more realistic challenge to microbial killing.

Biofilm was developed on the root canal walls of hemi-sectioned teeth. It is established that the biofilm forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria-substrate interaction on root canal walls (Kishen et al. 2006). In addition, exposure to dentine has been shown to have a powerful inhibitive effect on the antimicrobial effectiveness of several key disinfectants (Haapasalo et al. 2000). The downside of using dentine as a substrate is that the complexity and variability of the root canal system can represent an uncontrolled variable when evaluating antimicrobial agents. Results based on such models reflect a combination of the antimicrobial efficacy of the agent and its access to the biofilm (Spratt et al. 2001). To minimise the variability introduced through the complexity of the root canal system, the roots of extracted teeth were sectioned longitudinally to generate two flat slices incorporating half the periphery of the root canal in each section, increasing access of the test agent to the microbial load. Incorporation of fluid flow was a further feature designed to increase the clinical relevance of this in vitro model.
The period of growth of the biofilm was selected to achieve a dense and mature structure which evenly covered the substrate surface. Recent reports indicate that as the age of a biofilm increases, its susceptibility to antimicrobials decreases. Ozok et al. found that a 100-fold higher NaOCl concentration was needed to create a similar killing effect on aged biofilms than younger ones (Ozok et al. 2007). This increased resistance of aged biofilms can partly be explained by differences in biomass, with increased numbers of bacterial cells and more exopolymeric substance (Stewart et al. 2001).

Recent investigations of the topography and ultrastructure of biofilms of varying maturity indicated a time-dependant tendency for bacteria-induced corrosion of the dentine surface, production of a mineral precipitate and potentiation of biofilm mineralization (Kishen et al. 2006). The capacity of *E. faecalis* to form distinct calcified biofilms may be another mechanism that allows this microbe to persist in endodontically treated teeth. The maturity of the biofilm therefore appears to be a variable with significant clinical implications, warranting *in vitro* efforts to replicate its structure.
1.11 Aims

The aims of this study were:

1) To compare the efficacy of commonly used endodontic medicaments against *E. faecalis* cultured as a biofilm on dentine substrate. The medicaments tested were Ledermix paste, calcium hydroxide, Odontopaste, 0.2% chlorhexidine and 50:50 combinations of Ledermix/calcium hydroxide and Odontopaste/calcium hydroxide.

2) To compare the antimicrobial effect achieved through exposure to endodontic medicaments with that achieved by exposure to a constant concentration of sodium hypochlorite for varying times.
2. Materials and Methods

2.1 Organism

*E. faecalis* was stored as frozen stock cultures in 40% v/v glycerol at – 80°C. 100μL aliquots were used to inoculate 20mls of Todd Hewitt Broth (Oxoid Aust.). After overnight growth, the broth was used to inoculate the flow cells. Two strains of *E. faecalis* were used: a tetracycline resistant strain ATCC 51299, and a tetracycline sensitive strain ATCC 29212. Purity of cultures was monitored by periodic plating onto Todd Hewitt agar (Oxoid Aust.).

2.2 Agar diffusion test

Todd Hewitt Broth agar plates were streaked with the two strains of *E. faecalis*. Wells were punched out in the agar and filled with Ledermix paste. Zones of inhibition were evaluated after incubation for 48 hours.

2.3 Dentine sample preparation

One hundred dentine samples were produced from extracted teeth stored in thymol, which were decoronated and sectioned longitudinally to expose the inner walls of the pulp chamber and canals. They were then sectioned further to a size which approximately corresponded to the dimensions of the stainless steel coupons in the flow cell. Following sectioning, the smear layer and any pulpal tissue remnants were removed by treating the specimens with 17% EDTA, followed by 1% sodium hypochlorite for 4 minutes each. This was a modified disinfection protocol described by Haapasalo and Ørstavik, who used an ultrasonic bath and a 5.25% concentration of NaOCl (Haapasalo and Ørstavik 1987). The dentine slices were then allowed to air dry.
for 10 minutes, before being attached to the stainless steel coupons using Araldite adhesive.

![Materials and Methods](image)

**Figure 1.** Experimental set-up consisting of a nutrient reservoir, peristaltic pump, flow cell, and a waste vessel, all connected by sterile silicone tubing.

### 2.4 Biofilm growth

A biofilm was established using a continuous flow cell model based on that described by Dunavant *et al.* (Dunavant *et al.* 2006). The system consisted of a nutrient reservoir, a single channel flow cell, a peristaltic pump, and a waste vessel, all connected with silicone tubing. The flow cell temperature was maintained at 37°C using a heating bed.
The flow channel design incorporated circular recesses which allowed for the placement of 9 stainless steel coupons. Each coupon had a human dentine sample attached as described previously.

The flow cell, with coupons and dentine slices in situ, was then sterilised using ethylene oxide. The nutrient reservoir, silicone tubing, and waste vessel were sterilised by autoclaving at 121°C for 20 minutes and the system was carefully assembled aseptically.

Using the in-line peristaltic pump, the flow cell was filled with Todd Hewitt broth (Oxoid Aust.). The pump was shut off and the system was allowed to remain static for 48 hours to confirm sterility. After confirmation of sterility, 10 mls of an overnight broth culture was introduced into the flow cell through a syringe injection port upstream of the flow cell. The culture volume remained in the flow cell for 24 hours to allow for bacterial growth. Laminar flow was then established at a flow rate of 20ml/h. The volume of the flow cell was 50ml, making the dilution co-efficient 0.4 h⁻¹.

Preliminary experiments were conducted in order to determine an appropriate time to produce confluent adherent biofilms. The biofilm was developed in a low oxygen tension environment at 37°C, and two coupons were sampled weekly over four weeks for SEM analyses.
Materials and Methods

The flow cell accommodated steel coupons with a human dentine sample attached to each one. Once filled with media, *E. faecalis* ATCC51299 (Tetracycline resistant strain)

Figure 2. Flow cell showing coupons and dentine slices *in situ*

2.5 Scanning electron microscopy (SEM)

The dentine slices were stored overnight in fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS + 4% sucrose). They were then washed in PBS + 4% sucrose and dehydrated in ascending concentrations of ethanol as follows:

70% ethanol – 15 minutes

90% ethanol – 15 minutes

95% ethanol – 15 minutes

100% ethanol – 3 changes of 10 minutes each

Following critical point drying, the samples were coated with platinum and analysed under a scanning electron microscope (Philips XL30 field emission scanning electron microscope).
The biofilm was assessed for confluence, bacterial density and tubular penetration of the dentine substrate.

2.6 Test Agents Used

- 4% sodium hypochlorite (Asis Scientific, Adelaide)
- Ledermix (DENTSPLY, Australia)
- Odontopaste (Australian Dental Manufacturing)
- Calxyl – calcium hydroxide (OCO Préparate GmbH)
- Ledermix/calcium hydroxide
- Odontopaste/calcium hydroxide
- 0.2% chlorhexidine gel (Professional Dentist Supplies Pty. Ltd.)

Each test agent in paste or gel formulation was introduced individually into the flow cell, and applied to cover each dentine surface, while the control samples were removed from the flow cell and placed into sterile PBS for corresponding time periods. This was to prevent any antimicrobial effects from dissolved test agents on the control specimens. The dentine samples were exposed to each medicament for either 24 or 48 hours.

The sodium hypochlorite was in liquid form, and the testing regimen involved removing samples from the flow cell and submerging them in 2mls of sodium hypochlorite, while corresponding negative control samples were placed into sterile
PBS. The times of exposure were 1 minute, 10 minutes, 30 minutes and 60 minutes. Each protocol was performed in duplicate.

2.7 Determining Antimicrobial Efficacy

2.7.1 Biofilm Harvesting

Following exposure to the test agent or the control solution, each coupon was washed three times with sterile phosphate buffered saline (PBS) to remove residual test agent. Neutralising solutions were not used, as these were not available for all the agents tested and consistency in the protocol precluded its use for some but not all test agents. Following washing, the biofilm attached to each coupon was detached by sonication into 2 mls of sterile PBS. SEM analysis showed that <1% of cells remained on the dentine surface following sonication (data not shown).

To investigate the possibility that viable bacteria may reside in the dentinal tubule and were not removed by the surface sampling protocol, dentine samples were exposed to NaOCl for 10 minutes, 30 minutes and 60 minutes. They were then crushed using a sterile metal sleeve and cylinder, and suspended in 2 mls of sterile PBS. An aliquot (100μl) was placed into sterile saline and viable counts determined. The viable counts obtained through crushing the dentine samples could then be compared with viability counts obtained from surface sampling after sonication.
2.7.2 Cellular viability and protein measurement

Cellular viability was determined using serial plating onto Todd Hewitt agar plates (Oxoid Aust.). Viable counts were done in duplicate for each treatment procedure. Plates were incubated aerobically at 37°C for 48 hours, and the number of colony forming units (CFU) per ml was counted.

Cellular protein levels were measured to quantitate the amount of biofilm harvested. 0.9mls of the biofilm suspension used for viability counts was combined with 0.1mls of 1M NaOH, and boiled for 30 minutes. 150uL of each sample was pipetted into a microplate well, and combined with 150uL of the Coomassie Plus protein assay reagent (Pierce biotechnology). The microtitre plate was mixed for 30 seconds and the absorbance read at 595nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Protein concentrations were standardised against dilutions of bovine serum albumin (BSA) which were assayed in parallel with the unknown samples.

Colony forming units were then divided by cellular protein levels so that treatment protocols could be compared. Comparisons with the cellular viability in control biofilms (no treatment) allowed calculations of the percentage kill of bacteria following treatment with the test agents.
3. Results

3.1 Biofilm Growth

Preliminary experiments using scanning electron microscopy were conducted to determine an appropriate time to produce confluent adherent biofilms.

**Figure 3.** Scanning electron microscopy images showing biofilm structure at different stages of development. a) 1 week b) 2 weeks c) 3 weeks and d) 4 weeks

Figure 3 shows the development of the biofilm over 4 weeks. At week one, the cells appeared quite dispersed, with few dense aggregates. Interconnecting networks of polymer strands can be observed. At two weeks, denser cellular aggregates become apparent, but the coverage is not confluent. Three weeks post inoculation showed a
more confluent biofilm, with thick bacterial growth covering the dentine surface, leaving small areas of loosely organised cells. At four weeks, cells are present in dense, multi-layered, confluent aggregations, generally arranged in short chains. Uncolonised sections of dentine were absent.

On the basis of these findings, the growth period for subsequent biofilm development was maintained at 4 weeks.

3.2 Dentine surface prior to inoculation

To confirm the effectiveness of the disinfection protocol used to prepare the dentine sections, sample dentine surfaces were analysed under SEM (Philips XL30 field emission scanning electron microscope) and are shown in Figure 4. The surfaces appeared free of debris and microbes, and dentinal tubules appeared patent.

**Figure 4** SEM images of dentine surfaces following disinfection a) 8000× magnification b) 3500× magnification
3.3 Effective removal of bacterial biofilm

SEM was used to observe the efficiency of removing the biofilm from the dentinal surface (Figure 5). Following 5 minutes of sonication only a small number of cells remain on the tooth surface.

![Figure 5](image)

**Figure 5.** Dentine surface following the removal of a 4 week old biofilm using sonication.

3.4 Sodium Hypochlorite

The first experiment investigated the effect of 4% NaOCl on an *E. faecalis* biofilm using exposure times of 30 minutes, 60 minutes and 6 hours. Viable bacteria could not be cultured at any of these times compared to the control samples. An additional experiment was then conducted to further examine the relationship between the time of exposure and the level of bacterial killing. These experiments confirmed that total bacterial elimination occurred at 30 and 60 minutes (Fig. 6). However, it was also
discovered that a 95% drop in bacterial viability occurred at 1 minute, and this increased to 99.99% at 10 minutes of exposure.

*Viable cells expressed as colony forming units per microgram of protein

**Figure 6.** Effect of 4% sodium hypochlorite on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to 4% sodium hypochlorite for either 1 minute, 10 minutes, 30 minutes, 60 minutes or 360 minutes. The bacterial viability, measured as colony forming units per microgram of protein, was then determined for each time exposure. The data presented represents the averages of duplicate samples from 3 flow cells.

SEM was also used to determine the effect of NaOCl treatment on the dentinal surface. After exposure times of 10 minutes (Figure 7) and 30 minutes (Figure 8), dentine surfaces appeared free of microbes and debris. The dentinal tubules appeared patent.
**Figure 7.** Dentine surface following a 10 minute exposure to 4% sodium hypochlorite (800× magnification)

**Figure 8.** Dentine surface following a 60 minute exposure to 4% sodium hypochlorite (1000× magnification)
3.5 Crushed Samples

*E. faecalis* biofilm established over a 4 week period was exposed to 4% sodium hypochlorite for 10 minutes, 30 minutes and 60 minutes. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure, using both surface sonication and tooth crushing sampling methods. The tooth crushing method detected viable cells (86) after 30 minutes of exposure to NaOCl, whereas surface sampling did not detect viable cells (Fig. 9). Neither method demonstrated bacterial viability after 60 minutes of exposure to NaOCl.

*Viable cells expressed as colony forming units per microgram of Protein

**Figure 9.** Detection of viable *E. faecalis* cells in crushed and non-crushed dentine slices following NaOCl exposure for 10, 30 and 60 minutes. Data based on 3 samples.
3.6 Ledermix and Odontopaste

Ledermix and Odontopaste had no apparent effect on the cellular viability of a 4 week *E. faecalis* biofilm even after 48 hours of exposure to each medicament (Fig. 10 and 12). SEM analysis of the biofilm following exposure to Ledermix (Fig. 11) and Odontopaste (Fig. 13), showed an apparently undisturbed, thick biofilm covering the entirety of the dentine surface. 

![Effect of Ledermix Paste on E. faecalis biofilm viability](chart.png)

*Viable cells expressed as colony forming units per microgram of Protein*

**Figure 10.** Effect of Ledermix paste on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to Ledermix paste for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from 2 flow cells.
**Figure 11.** Dentine surface following 48 hours of exposure to Ledermix paste

**Figure 12.** Effect of Odontopaste on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to Odontopaste for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from a single flow cell.

*Viable cells expressed as colony forming units per microgram of Protein*
To assess the susceptibility of a different strain of *E. faecalis* to Ledermix paste, a tetracycline sensitive strain, ATCC 29212, was used to repeat the experiment. Despite the use of this strain, no reductions in viability were detected over the 48 hour experiment (Fig. 14). This was consistent with results from the agar diffusion test, which showed that Ledermix was unable to achieve zones of inhibition for either of the *E. faecalis* strains.
Viable cells expressed as colony forming units per microgram of Protein

**Figure 14.** Effect of Ledermix paste on tetracycline sensitive *E. faecalis* biofilm. *E. faecalis* biofilm established over a 4 week period was exposed to Ledermix paste for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from a single flow cell.

### 3.7 Calcium Hydroxide and 50:50 combinations of Calcium Hydroxide with either Ledermix or Odontopaste

Exposure to calcium hydroxide for 24 and 48 hours reduced the viability of *E. faecalis* by > 99.9% (Fig. 15). SEM images of the dentine surface following 48 hour exposure to calcium hydroxide show a disturbed biofilm, but with residual cells clearly present (Fig. 16). The high magnification image shows *E. faecalis* cells residing within the dentinal tubules.
*Viable cells expressed as colony forming units per microgram of Protein

**Figure 15.** Effect of calcium hydroxide on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to calcium hydroxide for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from a single flow cell.

**Figure 16.** Dentine surface following 48 hours of exposure to calcium hydroxide

a) at 2000×magnification, b) at 10000×magnification
Compared to the controls, calcium hydroxide combined with Ledermix and calcium hydroxide combined with Odontopaste, were able to reduce viability of *E. faecalis* by > 99.9%. This was similar to the microbial reductions achieved with the sole use of calcium hydroxide (Figure 17). SEM images of the dentine surface following 48 hour exposures to the Ledermix/calcium hydroxide combination (Fig.18) and the Odontopaste /calcium hydroxide combination (Fig.19), showed a disturbed biofilm, but with residual cells clearly visible.

*Viable cells expressed as colony forming units per microgram of Protein

**Figure 17.** Effect of endodontic medicaments on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to calcium hydroxide for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from 3 flow cells.
Figure 18. Dentine surface following 48 hours of exposure to a 50:50 Ledermix and calcium hydroxide (10 000× magnification)

Figure 19. Dentine surface following 48 hours of exposure to 50:50 Odontopaste and calcium hydroxide (10 000× magnification)
3.8 Chlorhexidine gel

Compared to the controls, 0.2% chlorhexidine gel reduced bacterial numbers by 97% after 24 and 48 hours of exposure (Fig. 20). SEM images of the dentine surface following a 48 hour exposure time showed a disturbed biofilm, but with residual cells clearly visible (Fig. 21).

*Viable cells expressed as colony forming units per microgram of protein

**Figure 20.** Effect of 0.2% chlorhexidine gel on *E. faecalis* biofilm viability. *E. faecalis* biofilm established over a 4 week period was exposed to calcium hydroxide for either 24 or 48 hours. The bacterial viability, measured as colony forming units per microgram of protein was then determined for each time exposure. The data presented represents the averages of duplicate samples taken from a single flow cell.
**Figure 21.** Dentine surface following 48 hours of exposure to 0.2% chlorhexidine gel (10 000× magnification)
4. Discussion

4.1 Antibiotic-containing medicaments

The results of this study indicate that antibiotic-containing medicaments alone have little quantitative effect on the viability of \textit{E. faecalis}. Exposure to either Ledermix paste or Odontopaste for up to 48 hours did not register any observable decreases in microbial numbers. SEM analyses found a complete inability of these medicaments to remove biofilm. This was consistent with recent findings by Norrington \textit{et al.}, who qualitatively examined mixed biofilms exposed to a variety of antibiotics (amoxicillin, clindamycin, metronidazole, doxycycline and azithromycin) for 3 and 8 days. Scanning electron microscope images showed that the antibiotics had no observable effect on the biofilms (Norrington \textit{et al.} 2008).

A major reason for the lack of effectiveness of either Ledermix paste or Odontopaste is the intrinsic resistance of \textit{E. faecalis} to several commonly used antibiotics, and perhaps more importantly, its ability to acquire resistance to all currently available antibiotics, either by mutation or by horizontal gene transfer (Sood \textit{et al.} 2008). A significant proportion of enterococcal isolates demonstrate acquired resistance to tetracyclines (Dahlén \textit{et al.} 2000; Geijersstam \textit{et al.} 2006) and intrinsic resistance to clindamycin (Singh \textit{et al.} 2002). Geijersstam noted that 57 of 59 endodontic isolates of \textit{E. faecalis} displayed the classic LS\textsubscript{A} phenotype which confers intrinsic resistance to streptogramin A and lincosamides such as clindamycin (Geijersstam \textit{et al.} 2006). Similarly, Dahlén \textit{et al.} found 25 out of the 26 \textit{E. feacalis} species isolated from root canals to be resistant to clindamycin (Dahlén \textit{et al.} 2000). In terms of tetracycline resistance, Dahlén \textit{et al.}
found 4 out of 29 enterococcal strains to be highly resistant to tetracycline, while Geijersstam et al. identified 17 tetracycline resistant isolates out of 59 (Geijersstam et al. 2006). The close proximity of bacterial cells within the biofilm readily allows the exchange of genetic information, including the transfer of antibiotic resistance. Two strains of *E. faecalis* with reportedly different levels of tetracycline sensitivity were used in this experiment to test the efficacy of Ledermix paste. No significant reductions in bacterial viability could be noted for either strain and this was supported by the lack of susceptibility to Ledermix paste by the two strains using the plate diffusion method. This may well be an indication that the level of tetracycline in Ledermix paste is too low to affect antibiosis. We were unable to source a strain of *E. faecalis* susceptible to clindamycin, an indication of how widespread resistance to this antibiotic is among enterococcal species.

In addition to the intrinsic or acquired antibiotic resistance of *E. faecalis*, the organisation of cells in a biofilm structure could be a further factor contributing to the lack of antimicrobial efficacy of Ledermix paste and Odontopaste. Cells growing in biofilms have been shown to be up to 1000 times more resistant to antimicrobial agents than their planktonically growing counterparts (Ceri et al. 1999). This was highlighted by Rossi-Fedele and Roberts, who identified the MIC of plate-grown *E. faecalis* to tetracycline to be 32 μg ml⁻¹. In the root canal model, where the microbe had presumably established a biofilm, cells survived irrigation with a tetracycline concentration of 30 mg ml⁻¹, almost 1000 times more than the concentration that had been effective in the plate culture (Rossi-Fedele and Roberts 2007).
Concern has been raised about the capacity of demeclocycline HCl in Ledermix to diffuse to the peripheral parts of dentine in sufficient concentrations to inactivate bacteria (Abbott et al, 1990). In this set of experiments, however, even the superficial surface of the dentine remained laden with *E. faecalis* biofilm seemingly unaffected by the antimicrobial effects of the antibiotic. This was also seen with the more recently introduced Odontopaste. Laboratory and clinical research is urgently needed to assess the properties of this new product. Its clinical benefit is likely to be derived from the potential anti-resorptive and sedative properties of the steroid component, rather than the antibacterial effects of the antibiotic, but this is yet to be supported by evidence, as are the manufacturers’ claims that it causes no dentinal discolouration (ADM).

### 4.2 Calcium Hydroxide

Our results showed that calcium hydroxide was able to achieve reductions in microbial viability of 99.9% at 24 and 48 hours of exposure. This finding was at variance with reports showing that the antimicrobial effectiveness of calcium hydroxide is abolished in the presence of dentine (Haapasalo et al 2000; Portenier et al 2001). This inconsistency in findings is most likely a reflection of methodological differences, with the present study involving sections of dentine rather than the vast active surface area of dentine in powder form. Consistent with other reports, calcium hydroxide was found to be unable to achieve total elimination of *E. faecalis* cells. The persistence of a small proportion of bacteria was confirmed through scanning electron microscopy. Many of the persisting cells were seen to be present in the protective environment of the dentinal tubules. Similar findings were noted when calcium hydroxide was combined with either Ledermix or Odontopaste. The combining of antibiotic based medicaments with
calcium hydroxide did not appear to elicit any synergistic action, nor did it diminish the effectiveness of calcium hydroxide when used alone. It is therefore unlikely that adding antibiotic based medicaments will improve disinfection within the root canal. Whether the high alkalinity of the calcium hydroxide influences the properties and diffusability of the steroid component of Ledermix or Odontopaste, and the therapeutic effect achieved through these combinations has yet to be adequately investigated.

The antimicrobial activity of calcium hydroxide is due to the release and diffusion of hydroxyl ions, which produce a highly alkaline environment incompatible with microbial survival. For calcium hydroxide to act effectively as an intracanal dressing, the hydroxyl ions must be able to diffuse through dentine and pulpal tissue remnants. Studies have demonstrated that hydroxyl ions derived from a calcium hydroxide medication do diffuse through root dentine, but the pH values decrease as a function of distance from the main canal (Nerwich et al. 1993). One of the limitations of our study was the relatively short time of exposure of *E. faecalis* to the calcium hydroxide containing medicaments. Forty-eight hours may not have been sufficient to allow hydroxyl ions to reach the deeper layers of root dentine. Other reports, however, have demonstrated the inability of calcium hydroxide to eliminate *E. faecalis* from dentinal tubules, even superficially, after incubation periods of 10 days (Haapasalo and Ørstavik 1987), and 7 days (Heling et al. 1992). It is, therefore, unlikely that total bacterial elimination would have been achieved had the exposure time been extended.

Abdullah *et al.* found calcium hydroxide to be equally effective against bacteria in a planktonic and biofilm presentation. Although this medicament was not able to achieve
100% elimination of *E. faecalis* cells, a greater than 4 minute exposure resulted in 99.9% bacterial reduction (Abdullah *et al.* 2005), a finding replicated by the present study. Much of the recent research has been critical of calcium hydroxide, yet in the context of a microbial biofilm (Abdullah *et al.* 2005) and in clinical studies (Byström *et al.* 1985; Sjögren *et al.* 1991; Shuping *et al.* 2000), it has been shown to be highly efficacious. A possible explanation for its efficacy against microbial biofilms is that calcium hydroxide is capable of dissolving organic tissue, and is therefore less inhibited by dense cellular aggregations and exopolysaccharide matrix. Tissue dissolution is a property that is neglected in investigations of antimicrobial efficacy, probably because it is redundant in study designs incorporating agar diffusion or bacterial suspension methods. In clinical endodontics, however, the capacity of a medicament to physically dissolve necrotic pulpal tissue or microbial colonies is critical to achieving disinfection.

Despite its ability to drastically reduce bacterial numbers, calcium hydroxide failed to achieve sterilisation of the dentine surface. One explanation for this is the well recognised capacity of *E. faecalis* to resist the antibacterial effect of calcium hydroxide (Haapasalo and Ørstavik 1987; Safavi *et al.* 1990; Siqueira and de Uzeda 1996). Byström *et al.* showed that the pH needs to reach levels of 11.5 or higher to exert a bactericidal effect on *E. faecalis* (Byström *et al.* 1985). Investigations of the mechanisms involved in the resistance of *E. faecalis* to calcium hydroxide identified that its survival at high pH was related to the effective function of its proton pump. When cells were exposed to calcium hydroxide at pH 11.1 for 30 minutes, there was a 20-fold reduction in cell survival in the presence of the proton pump inhibitor CCCP (Evans *et al.* 2002). Furthermore, the buffering effects of dentine may not allow a sufficiently high pH to be achieved in the dentinal tubules (Haapasalo *et al.* 2000). The
biofilm forming ability of *E. faecalis* constitutes another important survival strategy (Distel *et al.* 2002), while its proficiency at invading dentinal tubules affords the organism physical protection from chemomechanical root canal preparation and intracanal dressings (Love 2001).

### 4.3 Chlorhexidine gel

Chlorhexidine gel was able to eliminate 97% of bacteria at 24 and 48 hours of exposure, making it less effective than calcium hydroxide (99.9%), calcium hydroxide combined with Ledermix (99.9%) and calcium hydroxide combined with Odontopaste (99.9%). Chlorhexidine was also considerably less effective than sodium hypochlorite (100%), a consistent finding in biofilm studies. According to Dunavant *et al.*, 6% sodium hypochlorite achieved >99.99% elimination of *E. faecalis* cells after a 5 minute exposure, while 2% chlorhexidine achieved only a 60.5% bacterial reduction (Dunavant *et al.* 2006). In comparison to these findings, chlorhexidine performed considerably better in our experiment. This is most probably due to the longer exposure time, and the greater initial volume of biofilm to which the persisting bacterial load is related to in the calculations of percentage kill. Quantitatively, 3% bacterial survival may represent a very significant number if the initial bioburden was extensive. SEM images showed residual bacteria and exopolymeric material persisting on the dentine surface. This is consistent with findings by Clegg *et al.*, who noted a virtually intact biofilm after exposure to chlorhexidine, and suspected the antimicrobial of fixation (Clegg *et al.* 2006).
Chlorhexidine has consistently been found less effective than sodium hypochlorite in biofilm studies (Abdullah et al. 2005; Dunavant et al. 2006; Williamson et al. 2009). This is in contrast to in vitro experiments using broth dilution tests on suspended cells or in models where bacterial cells are used to inoculate an extracted root. Such study models generally find the antibacterial effectiveness of chlorhexidine and sodium hypochlorite to be similar (Gomes et al. 2001; Vianna et al. 2004; Oncag et al. 2003). In vivo studies have generated less favourable results when evaluating the antimicrobial efficacy of chlorhexidine. Many have found that the microbial reduction achieved through mechanical instrumentation and the use of an antimicrobial irrigant could not be improved upon with the use of an additional chlorhexidine medicament, and in some cases the number of recoverable microorganisms actually increased after a chlorhexidine dressing was placed (Manzur et al. 2007; Paquette et al. 2007). Vianna et al. compared the degree of microbial reduction after chemo-mechanical preparation using either sodium hypochlorite or chlorhexidine in vivo. Significantly greater bacterial reduction was achieved through the use of NaOCl compared to chlorhexidine (99.9% vs 96.6%) (Vianna et al. 2006).

The activity of chlorhexidine is pH dependent, and is greatly reduced in the presence of organic matter (McDonnell and Russel, 1999). Its inability to dissolve organic tissues is therefore a major shortcoming, and responsible for the reduced antimicrobial effectiveness of chlorhexidine when challenged by an increased bioburden. Sodium hypochlorite, on the other hand, acts as a solvent in the presence of organic tissue, releasing chlorine which, combined with the protein amino group, forms chloramines (Baumgartner and Cuenin 1992). This tissue dissolving ability is one of the features that makes sodium hypochlorite such an effective endodontic irrigant.
4.4 Sodium Hypochlorite

In this study, 4% sodium hypochlorite was the only agent capable of totally eliminating the *E. faecalis* biofilm. Its antimicrobial efficacy was time dependant, with a 95% drop in bacterial viability noted at 1 minute, 99.9% at 10 minutes, and no detectable bacteria cultured at 30 or 60 minutes. Crushing the dentine revealed viable cells after 30 minutes of sodium hypochlorite exposure, even though surface sonication failed to produce any colony forming units from the same dentine slice. This highlights the limitation of surface sampling methods in assessing disinfection, such as the paper point sampling used in clinical endodontics. A negative culture result is merely an indication that microbial numbers are below the point of detection, rather than a guarantee of sterility.

Scanning electron microscopy supported the quantitative findings, with small numbers of *E. faecalis* cells and polymeric matrix substance visible after 1 minute. Longer exposure times produced dentine walls free of microbial cells or debris, with patent dentinal tubules. Variable degrees of surface erosion were noted, and this appeared to be a function of time. Clinically this indicates that sodium hypochlorite is capable of both eliminating infecting microbes and physically removing them, but its action is time-dependant. Continuous replenishing with fresh solution and ultrasonic activation are mechanisms that may speed up the antimicrobial and tissue dissolving action of sodium hypochlorite (Sjögren and Sundqvist 1987; Spoleti *et al.* 2003), although this is yet to be shown against a bacterial biofilm. Consequently, the time required to exert its effect in the root canal may be reduced, limiting the structural damage to dentine.
4.5 Limitations and future research direction

The length of time to establish the experimental biofilm, the limited number of samples per flow cell and the need to include control and SEM samples in each cell limited the number of experiments that could be conducted within the study period. As such, the sample sizes are relatively small and preclude statistical analyses of the results. A flow cell capable of accommodating a greater number of specimens would be more efficient at achieving such statistical power. Attempts to use reverse transcriptase PCR (RT-PCR) to survey cellular viability in samples obtained with sodium hypochlorite were unsuccessful, and further work to streamline the experimental protocol relating to PCR is being undertaken. This would be particularly useful in investigating whether *E. faecalis* cells adopt the VBNC state as a function of antimicrobial agent exposure. Live/dead stain and quantitative (real time) PCR would also have contributed positively to this research. Further limitations of this study are that a bacterial monospecies was used to establish the biofilm. Microbiological studies using culturing techniques have frequently isolated an *E. faecalis* monoinfection in failing root canals (Sundqvist et al. 1998; Pinheiro et al. 2003), although this is more likely a reflection of the limitations of the sampling and culturing technique than the true state of infected post-treatment root canals.

The biofilm in our study received a continuous and plentiful nutrient supply in the form of Todd Hewitt Broth which flowed over the biofilm surface at a constant rate. It has been documented that certain biofilm characteristics such as density of growth and depth of dentine tubule penetration are influenced by the level of nutrition and oxygen concentration to which they are exposed (George et al. 2005). The growth environment
in the enclosed cell provided the cells with very low oxygen concentration, but repeating the experiment with a reduced nutrient supply may have altered some aspects of the biofilm structure and potentially influenced its susceptibility to the test agents. The dentine sections maximised the area of contact between the bacteria and the antimicrobials. An experimental model incorporating root canals of standardised sizes may have offered a greater antimicrobial challenge.

Saline controls throughout this study showed a measurable reduction in viable cells. This reduction can be attributed to the removal of the biofilm from its nutrient source, leading to the death of some of the cell populations. After 48 hours in nutrient-poor PBS this outcome was not unexpected. The osmolarity of the saline may have contributed to additional cell damage.

One of the potential concerns of using sonication to harvest the biofilm following exposure to test agents is that some cells may be physically damaged through this process. However, this is a protocol that has been used previously in biofilm research (Chai et al. 2007), without reports of cellular damage. Furthermore, high magnification SEM images in this study confirm the presence of intact cells post-sonication, indicating that any damaging influence of sonication must be minor.
5. Conclusions

When used in isolation, antibiotic containing medicaments had no appreciable effect on the viability of *E. faecalis*. Using a tetracycline sensitive strain did not improve the antimicrobial effectiveness of Ledermix paste. Calcium hydroxide in isolation, and when combined with Ledermix or Odontopaste, achieved a 99.9% reduction in *E. faecalis* viability, compared to 97% achieved by chlorhexidine gel. The antimicrobial effectiveness of sodium hypochlorite increased exponentially with increased exposure time. Total bacterial elimination was predictably achieved at 30 minutes of exposure. A comparison of sampling methods showed that crushing the dentine sample was able to identify bacterial viability in specimens deemed sterile through surface sampling.
6. References


7. Appendix

**Ledermix**

*E. faecalis* biofilm was exposed to Ledermix paste for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 1, 2 and 3.

<table>
<thead>
<tr>
<th>Time of exposure (hrs)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
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</tr>
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*Table 1.* Ledermix paste and tetracycline resistant strain ATCC 51299
<table>
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<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
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</thead>
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<td>13.33</td>
<td>$1.0 \times 10^5$</td>
<td>26</td>
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</table>

*Table 2.* Ledermix paste and tetracycline resistant strain ATCC 51299

<table>
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<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
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</tr>
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<td>50</td>
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<td>$9.8 \times 10^4$</td>
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*Table 3.* Ledermix paste and tetracycline sensitive strain ATCC 29212.
4% Sodium Hypochlorite

*E. faecalis* biofilm was exposed to 4% NaOCl for 30 minutes, 60 minutes and 360 minutes. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 4, 5, and 6.

<table>
<thead>
<tr>
<th>Time of exposure (minutes)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
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<td>100</td>
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<td>360 control</td>
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<td>15.38</td>
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*Table 4. Sodium hypochlorite*
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<th>Time of exposure (min)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
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**Table 5. Sodium hypochlorite**

<table>
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<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
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**Table 6. Sodium hypochlorite**
**Crushed Samples 10, 30, 60 min**

*E. faecalis* biofilm was exposed to 4% Sodium Hypochlorite for 10 minutes, 30 minutes, and 60 minutes. The number of colony forming units was calculated using both surface sonication and tooth crushing sampling methods. This was divided by the protein levels in order to determine viability. Percentage kill could then be calculated.

See Table 7

<table>
<thead>
<tr>
<th>Time of exposure (min)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
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**Table 7. Crushed vs Surface sampling**
Odontopaste

*E. faecalis* biofilm was exposed to Odontopaste for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 8

<table>
<thead>
<tr>
<th>Time of exposure (hours)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
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<td>58.9</td>
<td>$4.4 \times 10^4$</td>
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</tr>
<tr>
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<td>55.5</td>
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<td>86.4</td>
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</table>

*Table 8. Odontopaste*
Calcium Hydroxide

*E. faecalis* biofilm was exposed to calcium hydroxide for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 9.

<table>
<thead>
<tr>
<th>Time of exposure (hours)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.2 × 10⁶</td>
<td>16.4</td>
<td>3.8 ×10⁵</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.6 × 10⁵</td>
<td>8.3</td>
<td>5.5 ×10⁵</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>112</td>
<td>44.4</td>
<td>2.5</td>
<td>99.9</td>
</tr>
<tr>
<td>24</td>
<td>95</td>
<td>40.2</td>
<td>2.4</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>347</td>
<td>44.0</td>
<td>7.9</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>60</td>
<td>54.6</td>
<td>1.1</td>
<td>99.9</td>
</tr>
<tr>
<td>48 control</td>
<td>4.9 × 10⁶</td>
<td>48.3</td>
<td>1.0 ×10⁵</td>
<td>78.5</td>
</tr>
<tr>
<td>48 control</td>
<td>2.2 × 10⁶</td>
<td>29.3</td>
<td>7.5 ×10⁴</td>
<td>83.9</td>
</tr>
</tbody>
</table>

Table 9. Calcium Hydroxide
50:50 Ledermix and Calcium Hydroxide

*E. faecalis* biofilm was exposed to a 50:50 mixture of Ledermix and Calcium Hydroxide for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 10

<table>
<thead>
<tr>
<th>Time of exposure (hours)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$9.1 \times 10^6$</td>
<td>51.3</td>
<td>$1.8 \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$1.5 \times 10^7$</td>
<td>49.8</td>
<td>$3.0 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>93</td>
<td>65.8</td>
<td>1.4</td>
<td>99.9</td>
</tr>
<tr>
<td>24</td>
<td>145</td>
<td>86.7</td>
<td>1.7</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>120</td>
<td>50.3</td>
<td>2.4</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>240</td>
<td>73.6</td>
<td>3.3</td>
<td>99.9</td>
</tr>
<tr>
<td>48 control</td>
<td>$9.6 \times 10^5$</td>
<td>46.2</td>
<td>$2.1 \times 10^4$</td>
<td>91.3</td>
</tr>
<tr>
<td>48 control</td>
<td>$3.5 \times 10^6$</td>
<td>69.0</td>
<td>$5.1 \times 10^4$</td>
<td>78.8</td>
</tr>
</tbody>
</table>

Table 10. 50:50 Ledermix and Calcium Hydroxide
50:50 Odontopaste and Calcium Hydroxide

*E. faecalis* biofilm was exposed to a 50:50 mixture of Odontopaste and Calcium Hydroxide for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 11

<table>
<thead>
<tr>
<th>Time of exposure (hours)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$5.7 \times 10^6$</td>
<td>29.7</td>
<td>$1.9 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$3.1 \times 10^6$</td>
<td>60</td>
<td>$5.2 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>180</td>
<td>71.3</td>
<td>2.5</td>
<td>99.9</td>
</tr>
<tr>
<td>24</td>
<td>180</td>
<td>178.3</td>
<td>1.0</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>$4.15 \times 10^3$</td>
<td>54.7</td>
<td>76</td>
<td>99.9</td>
</tr>
<tr>
<td>48</td>
<td>$3.87 \times 10^3$</td>
<td>117.4</td>
<td>33</td>
<td>99.9</td>
</tr>
<tr>
<td>48 control</td>
<td>$1.76 \times 10^6$</td>
<td>122.5</td>
<td>$1.4 \times 10^4$</td>
<td>88.4</td>
</tr>
<tr>
<td>48 control</td>
<td>$1.02 \times 10^6$</td>
<td>73.1</td>
<td>$1.4 \times 10^4$</td>
<td>88.4</td>
</tr>
</tbody>
</table>

Table 11. 50:50 Odontopaste and Calcium Hydroxide
**Chlorhexidine gel 0.2%**

*E. faecalis* biofilm was exposed to 0.2% chlorhexidine gel for 24 and 48 hours. The number of colony forming units was calculated, and divided by the protein levels in order to determine viability. The percentage kill could then be calculated. See Table 12.

<table>
<thead>
<tr>
<th>Time of exposure (hours)</th>
<th>Viable count (cfu/ml)</th>
<th>Protein (µg/ml)</th>
<th>Viability (cfu/mg protein)</th>
<th>Percentage kill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.05 \times 10^6$</td>
<td>22.9</td>
<td>$4.6 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$2.9 \times 10^6$</td>
<td>38.7</td>
<td>$7.5 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>$6.1 \times 10^4$</td>
<td>50.7</td>
<td>$1.2 \times 10^3$</td>
<td>98</td>
</tr>
<tr>
<td>24</td>
<td>$9.5 \times 10^4$</td>
<td>52.5</td>
<td>$1.8 \times 10^3$</td>
<td>97</td>
</tr>
<tr>
<td>48</td>
<td>$1.23 \times 10^5$</td>
<td>51.6</td>
<td>$2.4 \times 10^3$</td>
<td>96</td>
</tr>
<tr>
<td>48</td>
<td>$7.25 \times 10^4$</td>
<td>79.4</td>
<td>$9.1 \times 10^4$</td>
<td>98</td>
</tr>
<tr>
<td>48 control</td>
<td>$5.9 \times 10^5$</td>
<td>38.5</td>
<td>$1.5 \times 10^4$</td>
<td>75</td>
</tr>
<tr>
<td>48 control</td>
<td>$7.4 \times 10^5$</td>
<td>65.6</td>
<td>$1.1 \times 10^4$</td>
<td>82</td>
</tr>
</tbody>
</table>

*Table 12. Chlorhexidine gel*