Investigation of the Effect of Rapid and Slow External pH Increases on *Enterococcus faecalis* Biofilm Grown on Dentine

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Endodontics)

September 2011

Dr Mark Stenhouse

BDS (Otago), FRACDS

THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Table of Contents

Abstract

Objectives: Calcium hydroxide is a common endodontic medicament that produces a localized rise in hydroxyl ion concentration. *Enterococcus faecalis* has shown some resistance to calcium hydroxide. The aim of this study was to compare the survival of an *E. faecalis* biofilm that had been grown on dentine when exposed to rapid or slow increases in external pH.

Method: A flow cell apparatus was used to grow single species *E. faecalis* biofilm on dentine discs. Following four weeks growth in Todd Hewitt Broth (THB), flow cells were exposed to either a rapid or slow increase to pH 11.5 or 12.5 using pH buffered growth medium. After four days exposure to pH 11.5 or 12.5, the flow cells were dismantled and the dentine discs were sonicated in saline solution to dislodge the attached biofilm. Viability of *E. faecalis* was established by serial dilution and plating onto THB agar plates. Viability was then normalised to total protein as determined by protein assay. Scanning electron microscopy (SEM) and confocal laser microscopy (CLSM) was also carried out to qualitatively observe the effects of the different rates of pH increase.

Results: A significant difference in viability between a slow or rapid increase in pH has not been shown by this study. pH 12.5 solutions were more effective at killing bacteria than pH 11.5 but even at this high pH some *E. faecalis* still survived. Exposure to high pH drastically reduced the numbers of bacteria observed on the dentine discs by SEM and CLSM although some did persist.

Conclusion: Based on the results of this study, *E. faecalis* located at sites within the root canal where a slower rise in pH is likely following application of a high pH medicament such as calcium hydroxide, do not seem any more likely to undergo an adaptive response that will increase their resistance and survival than the same bacteria in locations where the pH rise will be rapid. The demonstrated survival of *E. faecalis* in a high pH environment similar to that experienced clinically may help explain the problems associated with current treatment protocols when retreating root filled teeth.

Declaration

I, Mark Stenhouse, declare that this work 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. It contains no material which has been accepted for the award of any other degree of diploma in any university or tertiary institution.

I give consent to this copy of my thesis to be made available to the University Library, for loan or photocopying, subject to the provisions of the Copyright Act 1968, as well for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Declared by:_____ Witnessed by: _____

Mark Stenhouse

Date: _____ Date: _____

Acknowledgements

I would like to thank Associate Professor Peter Cathro for being so generous with his time and wisdom in support of this project and during my time in this DClinDent program. His vision, sense of calm and guidance has been invaluable in helping me to keep seeing the forest for the trees.

Without Dr Peter Zilm's contribution, this study would not have been possible. His knowledge of microbiology and expertise in scientific method have been invaluable to this work. Dr Zilm's ability to explain complex ideas in straightforward terms has been a godsend.

I would also like to acknowledge the assistance of the staff at the Adelaide Microscopy Centre and especially Ms Lyn Waterhouse for her patience, time and expertise.

Thank you to Professor Geoffrey Heithersay for being so generous with his wealth of clinical and scientific knowledge and experience. I have been very fortunate and grateful for his ongoing involvement in the endodontic program.

The support and friendship of my fellow DClinDent candidates, Dr Barbara Plutzer, Dr Aaron Seet, Dr Jonathon Christo and Dr Mohammed El Kishawi, has made the last three years a pleasure which I will always remember fondly.

To my wife Belinda, I cannot thank you enough for your patience, support and encouragement, even through my "stroppy" times.

Finally, I would like to acknowledge the financial assistance of the Australian Dental Research Foundation and Australian Society of Endodontology.

1.1 Introduction

One of the most cited papers in the endodontic literature is a landmark study by Kakehashi *et al.* (1965) that demonstrated the causal relationship of microorganisms to pulp necrosis. This was followed by several other microbiological studies establishing microbial infection as the primary cause of endodontic disease (Fabricius *et al*., 1982a, Möller *et al*., 1981). Microorganisms are not only a major cause of pulp necrosis but also initiate and maintain apical periodontitis. As a result of these findings the primary goal of endodontic treatment has become the elimination and prevention of microbial infections within the root canal system.

The three traditional pillars of endodontic treatment are instrumentation, irrigation and medication. With these three fundamentals of treatment, high rates of success have been achieved (Byström *et al*., 1987, Sjögren *et al*., 1990). However, during the last 20 years, much attention has focused on and questioned the value of medication within endodontic therapy. This has occurred because several studies have found that calcium hydroxide, the main medicament used in endodontics today, has a number of limitations and may not be as effective at disinfecting the root canal system as once thought (Haapasalo and Ørstavik, 1987, Peters *et al*., 2002). Indeed, several clinical studies have found no statistical difference in the outcome between treatment regimens using calcium hydroxide medication and treatments being completed in a single visit (Peters and Wesselink, 2002, Trope *et al*., 1999, Weiger *et al*., 2000).

Calcium hydroxide has a pH of 12.5 and thus exerts its antimicrobial effect by increasing the localized pH (Siqueira and Lopes, 1999). However, as it can only be delivered within the main prepared canal space it must rely on its own properties of diffusion to access more hard to reach areas of the root canal. These areas include accessory canals, dentinal tubules, isthmuses and ramifications that have been shown to harbour microorganisms (Nair *et al*., 2005, Peters *et al*., 2001a). As a result of such diffusion, in combination with a potential dentine buffering effect, the pH rise in such locations is slower than in

the main canal (Haapasalo *et al*., 2000, Nerwich *et al*., 1993). It would thus seem conceivable that such a slow rise may allow time for a degree of adaptation by some microbiota within the complex root canal system to the changing environment. Such adaptations may enhance their chances of surviving calcium hydroxide medication and additional insults associated with endodontic therapy.

Other aspects of root canal infections may also inhibit the effectiveness of calcium hydroxide medication. For example, some individual bacteria may be more resistant than others. In particular, *Enterococcus faecalis*, a bacteria commonly found in failed root canal treated teeth, has shown a particular ability to survive exposure to calcium hydroxide. Furthermore, it has become a widely accepted concept that the root canal microbiota largely exists as a biofilm (Nair, 1987, Ricucci and Siqueira, 2010, Siqueira, 2002). Biofilms give rise to a number of protective advantages which may be physical, physiological or genetic. As a result, the therapeutic dosage required of any medicament may need to be increased when bacteria such as *E. faecalis* are present as a biofilm (Svensåter and Bergenholtz, 2004).

The primary question this literature review and subsequent research will investigate is whether variation in the rate of increase in external pH will affect the survival of *E. faecalis* when grown using an *in vitro* biofilm model.

1.2 Microbiological Basis of Endodontic Disease

The research of Kakehashi *et al.* (1965) is regarded as the first to show irrefutably that bacteria are the key agent in pulpal necrosis. This was achieved by exposing normal and germ-free rats to a surgical pulp exposure. They subsequently treated and fed the rats the same way except that the germ-free animals were maintained under sterile conditions. As a result, despite the persistent trauma of food impaction, the germ-free animals maintained vital pulps and healthy periapical tissue. Healing occurred with evidence of dentine bridge formation. In contrast, the control rats all suffered pulp necrosis.

Moreover, a periapical infiltration of chronic inflammatory tissue and abscess formation occurred only in control rats.

While Kakehashi *et al.* (1965) showed that bacteria were a causal agent of pulp necrosis, the role of bacteria in apical periodontitis was less definitive. Although periapical disease was only found in the conventionally infected rats it was still possible that the periapical component of endodontic disease may be due to the breakdown products of the necrotic pulp rather than the microorganisms that caused the pulp necrosis.

Sundqvist (1976) partially addressed this question left by Kakehashi *et al.* (1965). He examined 27 patients with 32 teeth that had been traumatized and subsequently become non-vital. Of these 32 teeth, 19 had apical periodontitis associated with them while the other 13 did not. Using careful sampling techniques of the root canal, it was shown that 18 out of the 19 teeth that had apical periodontitis also produced positive bacterial cultures. In contrast, the other 13 teeth without lesions sampled negative for the presence of microorganisms. Therefore, apical periodontitis only seemed to be associated with infected canals suggesting that bacteria were responsible for this disease. This study is particularly significant because Sundqvist used a comprehensive range of new protocols in order to provide the most accurate picture of endodontic infection that had ever been published. This was considered a particularly robust, thorough and accurate microbiological study for the time and remains so today.

In another Swedish study, Möller *et al.* (1981) continued what was becoming a series of well-designed and complementary studies implicating bacteria as the causal agent of endodontic diseases. While Kakehashi *et al.* (1965) had definitively shown that bacteria caused pulpal disease and Sundqvist (1976) had shown apical periodontitis only occurring in the presence of bacteria, a temporal relationship between pulpal infection and apical periodontitis in a previously healthy patient had yet to be shown. Thus, Möller *et al.* (1981) carried out an experiment amputating pulps of previously sterile root canals in monkeys and infecting the canals with indigenous bacteria from their saliva. They compared

the clinical and radiographic results with another group of canals that had had their pulps amputated but were kept sterile (Möller *et al*., 1981). All teeth were initially vital and without apical periodontitis. After pulp amputation, with or without infection, all teeth were sealed for 6-7 months. Upon reopening, the group of teeth with sterile amputated pulps had remained necrotic and no bacteria could be isolated from them. Significantly, none of these teeth had any clinical or radiographic signs of apical periodontitis. In contrast 47 out of 52 teeth that had been infected developed radiographic lesions representing apical periodontitis. The inflammatory changes in the apical region of the infected group could only be explained by the contamination with infected saliva. This work, therefore, dispelled the possibility that a necrotic but uninfected pulp may be capable of causing apical periodontitis although some question could still be asked as to the influence of non-microbial factors in saliva.

Fabricius *et al.* (1982) subsequently conducted an experiment using eight species of bacteria sourced and cultured from a necrotic lateral incisor of a monkey. They infected the root canals of 12 teeth from other monkeys and sealed them for six months. After re-accessing these teeth, bacteria were re-isolated from all the canals. All 12 teeth also showed radiographic development of apical periodontitis. What was most interesting however was the fact that despite being introduced in equal numbers, the eight strains changed their relative proportions to match the monkey from which they had originally came. This demonstrated that selective growth pressures existed in the root canal and suggested a degree of microbial interaction. The fact that the bacteria had re-established themselves into the same relative proportions as seen in the animal from which they had originally been cultured suggested that a specific community of bacteria are responsible for disease rather than a specific micro-organism.

1.2.1 Microbiological Identification- Culturing vs Molecular Techniques

The preceding studies discussed in Section 1.2 all utilized culture techniques to grow and identify bacteria taken from root canal samples. That is, they used a variety of nutrient media under both aerobic and anaerobic conditions to grow colonies of sampled bacteria on that media. A range of techniques based on phenotypic characteristics such as colony morphology and biochemical tests were then used to identify individual species. They used what were the most up-to-date techniques for that time and their findings still remain widely cited and undisputed today. Culture techniques are still widely used in microbiology although with many modern advances in the methodology and tests applied to identify different species.

However, the last decade has also seen the rise of molecular techniques in the study of endodontic microbiology. Molecular techniques generally refer to variations of polymerase chain reaction (PCR). PCR enables the amplification of only few a strands of DNA into millions of strands that can be detected and analyzed in a number of ways to specifically identify different species. There are a number of variations of PCR techniques that can be used depending on the information required. For example, Reverse Transcriptase PCR (RT-PCR) can identify the presence of mRNA within a sample and thus the expression of certain genes. Real Time-PCR uses fluorescent probes to detect and quantitate the DNA strands being replicated during the PCR process and thus quantify a certain species within a sample. Molecular techniques have contributed to and changed the way in which we currently view the findings of the culture studies that have been discussed so far. This new knowledge has generally either confirmed or added to the findings of culture studies (Siqueira and Rôças, 2010).

Both culturing and molecular techniques have their pros and cons. In reality, both techniques have their place in microbiological analysis of endodontic infections as long as their limitations are recognized and acknowledged. A problem common to both techniques is recovery of representative samples. Studies by Vertucci (1984) and Peters *et al.* (2001) have shown the complexity of

the root canal system and the ability of bacteria to invade even dentinal tubules. No matter how diligent a sampling technique, bacteria within such hard to reach areas are unlikely to be recovered. Should certain bacteria preferentially favor these areas over the main canal it is conceivable that these bacteria may not even be sampled in the first instance.

Sathorn *et al.* (2007a) highlighted some of the concerns regarding the limitations of culturing techniques and therefore the relevance of microbial sampling to clinical outcome. The main limitation of culturing techniques is that many species of bacteria are simply not cultivable. Successful cultivation requires knowledge of the appropriate nutrients, environment and possibly even other microbial interactions necessary for a certain species to grow (Sundqvist and Figdor, 2003). Although over the last 40 years our knowledge has increased remarkably with regard to certain bacteria, the problem with complex cultivation environments is that we do not always know what we do not know and thus progress can be slow and difficult. In contrast, the sensitivity of molecular techniques is remarkable and as long as an organism is picked up in a sample it is possible to identify it.

Identification of cultured organisms is also difficult. Traditionally, a range of different characteristics and biochemical tests were used that were generally based on phenotypic characteristics (Holdeman, 1975). However, because phenotypic characteristics change depending on stress, growth conditions and microbial interactions, this may lead to ambiguous characteristics that make identification less predictable than desired. Modern techniques such as mass spectrophotometry are more rapid and accurate than traditional biochemical methods as they can identify the presence of specific proteins within samples. Nonetheless, the identification of different species by molecular genetic techniques is not based on phenotype but on unchanging genetic sequences and can thus be considered the gold standard in identification (Siqueira, 2003). The scenario of a culture technique to grow bacteria while using molecular techniques for identification is a good example of how the two techniques should be used together.

PCR also has significant limitations. The detection of certain sequences does not necessarily indicate viable bacteria (Nair, 2004). Young *et al.* (2007) found *E. faecalis* DNA persisted one year after cell death. In terms of interpreting molecular studies, this means that not all identified species are necessarily living. Instead they may have been transient bacteria unable to survive the unique conditions of the root canal. Thus, detection of such species may give a false impression of the make-up of the endodontic microflora. In contrast, with culturing techniques a colony-forming unit (CFU) is definitive evidence of viable bacteria.

Unless specific techniques of PCR are used such as Real Time-PCR or Reverse Transcriptase PCR, molecular techniques do not give any impression of quantification. As such, bacteria present in very low numbers, and thus possibly providing very little pathogenic effect to a microbial community as a whole, can be mistaken for a significant contributor to the endodontic milieu (Sundqvist and Figdor, 2003). Again, this issue does not arise for culturing techniques as CFU counts can be determined with serial dilutions and thus species can be quantified.

From the above discussion identifying only a few limitations of each technique, it is evident neither is perfect but also that they may complement each other in different ways. Thus, neither technique should be discounted and ways in which each can be used in complementary fashion should be explored.

1.2.2 Microbiology of the Root Canal

Within the oral cavity, 700 bacterial species have been identified (Aas *et al.*, 2005). In contrast the unique and demanding environment of the root canal only selects for a relatively special few species. Infected root canals have rarely cultured more than ten species per canal (Sundqvist, 1992). Using molecular techniques Sassone *et al.* (2008) has reported an average 26 species per canal in symptomatic cases and 20 per canal in asymptomatic cases. This is clearly an increase in the number of species per canal compared with culture studies but

still indicates the existence of selective pressures within the root canal given the much greater variety of species present in the oral cavity. Low oxygen tension and nutrient limitations are the most obvious selection pressures found in the root canal compared with the oral cavity. Given the influence of the environment in bacterial selection it is also not surprising that differences exist in the bacteria found in untreated necrotic root canals (primary infections) and failed endodontically treated teeth (persistent infections).

1.2.2.1 Primary Infections

In 1976 , Sundqvist published a very comprehensive study into the microbiology of untreated root canals, or primary infections. He used strict protocols for inclusion, sampling technique, anaerobic growth and identification in order to culture and detect even the most fastidious organisms present within the samples taken. While only 6-10 species were typically isolated per canal, 88 different species were identified from all the canals tested. 90% of these strains were obligately anaerobic. Only five strains grew in air.

Sundqvist's (1976) work had reported the greatest proportion of obligate anaerobic bacteria in the root canal system up to that point in time but this proportion has also not been bettered since. One issue with this study is that the sample teeth were all intact, without caries or restorations. They had been traumatized and secondarily infected. However, necrotic pulps with apical periodontitis are commonly associated with caries or failing restorations. It is possible that the cause of the infection may influence the type of micro-organisms that colonise the root canal. Subsequent microbiological studies using less stringent inclusion criteria have not reported such large proportions of anaerobic bacteria. Incidences of anaerobic bacteria have been more in the range of 65-75% (Baumgartner and Falkler Jr, 1991, Chu *et al*., 2005, Gomes *et al*., 2004). Sundqvist's (1976) work clearly showed the importance of anaerobic bacteria with respect to endodontic disease but it should be borne in mind that in more typical clinical situations, while obligate anaerobes still dominate, facultative anaerobic bacteria are also present.

From a biological perspective it makes sense that anaerobic bacteria are more common in necrotic infected root canals particularly when still sealed by dentinal walls and even by restorations. Oxygen supply must be limited, or at the very least rapidly consumed upon entry by aerobic bacteria and thus, an ecological niche is developed for anaerobic species. Fabricius *et al.* (1982) showed this and ruled out the possibility that other features of anaerobic bacteria may be responsible for their prevalence within the root canal such as possibly better initial adhesion and migration abilities. Three monkeys had their anterior teeth exposed to the oral environment for seven days. Samples were taken at the end of seven days and then the teeth were sealed for either 90, 180 or 1060 days. Initially, after seven days, the ratio of obligate anaerobes to aerobic strains was 1.6 : 1. However, the more remarkable results came after the teeth were re-entered at later times. At 90 days this ratio changed to 3.5 : 1, 6.5 : 1 at 180 days and 11.3 : 1 at 1060 days. These results indicate the elimination of aerobic species within sealed root canals and subsequent dominance of anaerobic species. Concurrent increases in the overall proportion of total bacterial cells also occurred, with anaerobic cells constituting 50-55% of the total flora at seven days, 85% at 90 days and 95-98% at 180 and 1060 days. These results show that even though some aerobic bacteria do initially inhabit the exposed root canal, once it is sealed and oxygen largely eliminated they clearly do not persist as well as obligately anaerobic species. In other words, the sealed canal provides an ecological niche much better suited to anaerobic bacteria than aerobic bacteria. This study also showed that the microbiota within the root canal is dynamic.

With our increased understanding of biofilms, it is the microbial communities that appear to be more significant than individual species of bacteria in primary root canal infections as will be discussed in Section 1.4. On the other hand, an appreciation of member bacteria may aid in determining and describing interactions and features of broader biofilm communities. *Fusobacterium, Prevotella* and *Peptostreptococcus* spp. have been the most common obligate anaerobes isolated by culture studies while *Streptococcus* spp. are the most common facultative anaerobes. *Porphyromonas* spp. have also been commonly

associated with symptomatic infections (Baumgartner and Falkler Jr, 1991, Gomes *et al*., 2004, Sundqvist, 1976).

The findings of these culture studies still hold true when modern molecular techniques are applied. However, molecular techniques have greatly expanded the number of species identified from root canals. Molecular techniques are the only method with which to reliably establish the presence of bacteria that are difficult or impossible to culture. Over 400 different bacterial species have now been identified from primary infected root canals and 45% of these have been reported exclusively by molecular techniques (Siqueira and Rôças, 2010). In a study by Sakamoto *et al.* (2006), 55% of the species identified from primary infected canals were uncultivable. *Fusobacterium, Peptostreptococcus* and *Prevotella* species were still amongst the most common species found but added to these were species of *Dialister, Eikenella, Tannerella* and *Treponema* (Sakamoto *et al*., 2006). Previous culture and microscopic studies had only found spirochaetes in a relatively small proportion of infected canals, whereas molecular techniques have them in as many as 78% of cases, with *Treponema denticola* now being considered one of the major species associated with symptomatic infections (Rôças *et al*., 2003). With molecular analyses, *Prevotella* and *Porphyromanas* species have been significantly associated with symptomatic infections, similar to culture studies, but so also have *Dialister, Tannerella* and *Treponema* (Siqueira, 2005). Based on prevalence alone, molecular techniques seem to show less variation between symptomatic and asymptomatic cases. This would suggest that it is the relative proportions of bacteria that may be significant in these cases rather than simply the presence or absence of individual species.

1.2.2.2 Persistent Infections

Persistent infection, or re-infection, of endodontically treated teeth differs from primary infections. The number of species that are generally identified per canal is much less in persistent infections, and culture studies have found mono-species infections may be quite common (Sundqvist *et al*., 1998, Molander *et al*., 1998). Also, the types of species differ with obligate anaerobes no longer being

as dominant as they are in primary infections. One bacteria in particular, *E. faecalis,* so ubiquitous in the endodontic literature, is also much more commonly found in cases of persistent or secondary infections compared with primary infections.

Up until the late 1980s investigations into the causes of failed root canal treated teeth were sparse. In 1990, Nair *et al.*, examined the root apices and periapical lesions of treated teeth with persistent periapical lesions using transmission electron microscopy (TEM). They found that six out of nine cases had evidence of intra-radicular bacteria remaining in the apical root canal and concluded that it was likely that persistent periapical lesions were a result of persistent intra-radicular infection. Of these six cases, four had bacteria present while the other two cultured yeast organisms. Even with such compelling evidence for the presence of intra-radicular bacteria in cases of persistent apical periodontitis and with the knowledge that it was the unique environment of the root canal that selected for certain organisms capable of surviving, it was not until 1998 that the first modern microbiological studies examined the microbiota associated with persistent infections of endodontically treated teeth. Not surprisingly, given the logical difference in conditions present between an untreated canal and an obturated canal, a distinct difference in microbiota was noted.

Two studies that were published in 1998 took samples from teeth with persistent apical periodontitis and showed similar findings (Molander *et al*., 1998, Sundqvist *et al*., 1998). Unlike Sundqvist's (1976) work and most other studies since on primary endodontic infections, bacteria were not isolated from every root canal. Only 68% of canals in Molander *et al.* (1998) and 44% in Sundqvist *et al.* (1998) showed positive bacterial cultures. It is easy to speculate that a certain percentage of false negatives are likely in such studies given that mechanical disruption and cleansing by files must occur to remove gutta percha. This may reduce some microbes to undetectable levels or introduce factors such as oxygen that may render some particularly fastidious organisms uncultivable

or dead. Indeed, Molander *et al.* (1998) showed the use of chloroform had a likely negative impact on microbial detection.

The major finding of these studies was that Gram positive facultatively anaerobic species dominated the microflora. They comprised 69% of the microbiota in the study by Molander *et al.* (1998), thus differing from the typical dominance of obligate anaerobes in primary infections. This is somewhat surprising from an oxygen tension perspective in that a treated root canal would seem likely to have as low an oxygen tension, if not lower than, an untreated canal. Therefore, it would seem unlikely that it is oxygen tension that is selecting for facultative bacteria in the treated root canal. The treated root canal is also unlikely to have an increase in carbohydrate nutrients which are generally favoured by facultatively anaerobic species over their obligate counterparts (Sundqvist and Figdor, 2003). Thus, the two dominant environmental traits within the root canal seem less significant in the selection of specific bacteria inhabiting the treated root canal. Other factors or traits of these bacteria must be involved that provide some survival advantage. For example, they may be more resistant to initial anti-microbial procedures of endodontic therapy or are more capable of adapting to changing environmental conditions.

The number of species present per canal is also lower in persistent infections than is generally found in primary infections. Molander *et al.* (1998) found only one or two strains in 85% of cases in which bacteria were isolated while Sundqvist *et al.* (1998) found the same number in 96% of cases. Given that the greater the number of species present has been associated with increased incidence of symptoms it is not surprising that persistent apical periodontitis is rarely symptomatic (Sundqvist, 1976). This low number of species may also provide some clues as to the selection of bacteria present because those bacteria less suited to microbial competition and yet self-sufficient metabolically may be more likely to survive and thrive. As such, certain microorganisms have been shown to occur much more frequently in failed endodontically treated root canals than in untreated canals. Most conspicuously, *Enterococcus faecalis* was

found be the most prevalent species in the studies of both Molander *et al.* (1998) and Sundqvist *et al.* (1998).

In contrast to the culture studies of Molander *et al.* (1998) and Sundqvist *et al.* (1998), the molecular techniques used by Sakamoto *et al.* (2008) identified bacteria in 100% of the samples from treated root canals with persistent periapical lesions. This was probably due to the increased sensitivity of these techniques. Again, the number of bacterial species per canal in persistent infections is higher when molecular studies are compared with culture studies similar to the difference seen in primary infections. Molecular techniques have found an average of five species per canal as compared with one or two species per canal using culture techniques (Siqueira, 2005). Molecular analysis has also confirmed the findings of culture studies that a higher prevalence of *E. faecalis* is found associated with persistent infections compared with primary infections (Rôças and Siqueira, 2004). However, other species have also been reported in much higher prevalence than previously thought and could be considered major suspects in persistent periapical disease such as *Pseudoramibacter alactolyticus, Propionibacterium propionicum and Filifactor alocis* (Siqueira and Rôças, 2010).

1.2.3 Endodontic Therapy

Management of endodontic disease can be clearly seen from the preceding discussion to be management of root canal infections. Based on the initial diagnosis this may encompass prevention or elimination of infection. If both these goals are achieved, high success rates have been observed when appropriate long-term follow-up has occurred (Sjögren *et al*., 1990). Success can be defined as the resolution of apical periodontitis or prevention of the development of apical periodontitis (Ng *et al*., 2008). In cases of primary endodontic infection, high quality treatment can result in greater than 90% success rates, while in cases of retreatment success rates are reduced to 60-70% (Sjögren *et al*., 1990, Sundqvist *et al*., 1998).

The three main pillars of endodontic therapy have traditionally been instrumentation, irrigation and intracanal medication.

Instrumentation is a mechanical debridement of the main root canal. This process has a two-fold function. Firstly, instrumentation has been shown by Byström and Sundqvist (1981) to generally reduce the microbial load by two or three orders of magnitude. However, even in relatively easy straight canals, instrumentation alone can only completely eliminate bacteria in approximately 20% of cases (Byström and Sundqvist, 1981, Dalton *et al*., 1998). In root canals with a more complex anatomy, such as the mesial roots of lower molars, elimination by instrumentation alone would be even less likely. Several studies have shown the impossibility of even instrumenting all parts of the main canal let alone other more complex features such as isthmuses (Peters *et al*., 2001b, Wu *et al*., 2000). The second function of instrumentation is to provide a convenient shape for the application of irrigation, medication and obturation. The shape should be of such a size that it maintains as much dentine as possible but also allows penetration of the irrigating needle close to the apex, which will enhance cleansing of the canal system (Sedgley *et al*., 2005c).

The second phase of therapy is irrigation. The most commonly used irrigant is sodium hypochlorite which has a two-fold function. Firstly it is antimicrobial, acting as a very effective nonspecific antiseptic (Dunavant *et al*., 2006). Secondly, it can dissolve organic tissue including biofilm (Beltz *et al*., 2003, Gordon *et al*., 1981). As irrigants are able to reach areas of the root canal system that mechanical instrumentation is unable to, the use of sodium hypochlorite will further increase bacterial reduction. The concentration reported in the literature for sodium hypochlorite is in the range of 0.5-5% (Byström and Sundqvist, 1983, McGurkin-Smith *et al*., 2005). Arguments for different concentrations are made based on antibacterial action, tissue dissolution and safety. However, regardless of concentration, *in vivo* studies have consistently found that instrumentation and irrigation consistently render 50-70% of canals bacteria free (Byström and Sundqvist, 1983, Shuping *et al*., 2000, Sjögren *et al*., 1997, Sjögren *et al*., 1991). However, this still leaves 30-40% of canals with residual bacteria, which has been shown to reduce the chances of success in these teeth (Sjögren *et al*., 1997).

Traditionally the third phase of endodontic treatment is the empirical placement of an intracanal inter-appointment medicament to help control this residual

infection. Calcium hydroxide has been the most commonly used medicament due to its *in vivo* efficacy and safety (Byström *et al*., 1985, Shuping *et al*., 2000, Sjögren *et al*., 1991). However, recent studies have questioned the value of such a dressing after finding no difference in successful outcomes between teeth treated in two appointments with a calcium hydroxide dressing and those treated in a single visit after only instrumentation and irrigation (Peters and Wesselink, 2002, Sathorn *et al.*, 2005, Sathorn *et al.*, 2007b, Trope *et al*., 1999, Weiger *et al*., 2000).

One important point that is not widely discussed and has only been reported in some but not all studies is that of microbial load (Peters *et al*., 2002, Shuping *et al*., 2000). Given the complicated anatomy of the root canal system it seems unlikely that a sterile canal is indeed a reasonable goal of endodontic treatment and perhaps reducing levels as far as possible is more realistic. The question therefore arises as to what are the minimum numbers of bacteria that would allow healing. The answer to this question is not known nor is what would become of a residual infection left behind after endodontic treatment. This is an important gap in scientific knowledge and is relevant to the use or not of calcium hydroxide. From the equivocal results of the previously mentioned outcome studies it may be interpreted that instrumentation and irrigation reduce bacterial load sufficiently to allow healing although scientific logic would suggest that reducing this load further with calcium hydroxide would be even more beneficial.

1.3 *Enterococcus faecalis*

Enterococcus faecalis is one of the most widely studied microorganisms in endodontic research. The main reasons for this is its high prevalence and proportion in root filled teeth suffering persistent apical periodontitis, its demonstrated resistance to treatment techniques and its ease of handling in the laboratory situation (Molander *et al*., 1998, Sundqvist *et al*., 1998). Given that the number of bacterial species per canal in teeth suffering persistent apical periodontitis is low and *E. faecalis* has often been identified as a sole isolate, it is often extrapolated that *E. faecalis* is a major aetiological factor in causing this disease (Sundqvist *et al*., 1998).

Enterococci are Gram positive and mostly facultative anaerobes. They are a particularly resilient bacteria able to grow in rather extreme conditions- temperatures ranging from 10-45 deg C, salinity of 6.5% and at high pH over 9.6 (Portenier *et al*., 2003). Enterococci have a range of intrinsic and acquired virulence factors that allow these bacteria to survive adverse conditions and then thrive when the right environmental conditions present themselves. Acquisition of a number of resistance factors to various antimicrobial agents makes this a particularly problematic bacteria in a range of medical environments (Sedgley *et al*., 2004).

As their name suggests Enterococci are commonly found in the gastrointestinal tract. However, they are a ubiquitous and opportunistic bacteria known to be pathogenic not only in treated root canals but also in urinary tract infections, abdominal infections and cases of endocarditis. The incidence of Enterococci causing nosocomial infections is on the rise and is a real concern given their ability to develop resistance and cause life threatening disease such as endocarditis (Portenier *et al*., 2003).

A number of species come under the genus Enterococcus - *faecalis, faecium, galinarum, hirae* to name a few. However, by far the most common species is *E.*

faecalis which accounts for 80% of Enterococcal infections. In endodontics, *E. faecalis* accounts for close to 100% of cases in which Enterococcus is found (Sedgley *et al*., 2005b, Siren *et al*., 1997).

1.3.1 *E. faecalis* in the Oral Cavity

For *E. faecalis* to invade root canals it must at some stage be present in the oral cavity. Surprisingly, few studies have looked at such a presence and those that have, have given variable results.

Gold *et al.* (1975) investigated the presence of Enterococcus spp. in the oral cavity. They found 60-75% of subjects had Enterococci isolated from one or more sites within the oral cavity. The tongue, vestibular mucosa, plaque, saliva and carious lesions were all tested. However, the total numbers were low relative to total bacteria isolated. *E. faecalis* was the most common species, recovered in 47% of individuals tested (Gold *et al*., 1975). However, the results of Gold *et al.* (1975) contrast with subsequent papers which have not found such a high prevalence of Enterococci.

More recently, Sedgley *et al.* (2005) carried out an of investigation looking at this phenomenon with quite different results. They used more modern molecular techniques in addition to traditional culturing methods to ensure the greatest sensitivity to *E. faecalis*. Even though molecular techniques are more sensitive than culturing techniques the prevalence was still much less than the Gold *et al.* (1975) study. With culturing, only 7% of saliva samples revealed *E. faecalis* while with molecular techniques this increased to 17% (Sedgley *et al*., 2005a). However, similar to Gold *et al.* (1975), the relative proportion of *E. faecalis* to total bacteria was very small and no more than 0.005%.

In another study 100 samples were taken from two different populations (Sedgley *et al.,* 2004). These were a group undergoing endodontic treatment and a second group without a need or history of endodontic treatment. Each group contained 100 participants. Those undergoing treatment had an *E. faecalis* prevalence of 11% compared with 1% of the non-treatment group. This

difference was statistically significant and suggests *E. faecalis* may either predispose a patient to the need for endodontic treatment or that some factor associated with endodontic disease or root canal treatment may favour *E. faecalis* (Sedgley *et al*., 2004). What was also interesting in this study was that all Enterococcus spp. discovered were *E. faecalis* .

Lastly, in 2006, Sedgley *et al*. carried out a similar study to Gold *et al*. (1975) by sampling a range of locations within the oral cavity for *E. faecalis*. The main differences were that Sedgley *et al* (2006) sampled a population of endodontic patients and used molecular identifcation techniques. This study revealed a much greater prevalence of 70% for *E. faecalis* when using PCR than the previous studies mentioned. Clearly this is a much closer prevalence to that which Gold *et al*. (1975) found. Oddly, when culture results were carried out, as had been used by Gold *et al*. (1975), *E. faecalis* was only found in 10% of oral cavities. Molecular techniques are much more sensitive than culture techniques but when these techniques are used bacterial viability cannot always be confirmed and nor can the microbial load always be quantified. These are both important aspects of microbial identification.

From these studies there are obviously contrasting results as to the prevalence of *E. faecalis* within the oral cavity. However, they were mostly looking at *E. faecalis* presence from the perspective of a permanent commensal organism. Another possibility may be that *E. faecalis* is a transient organism in the oral cavity. Such an explanation may shed light on the variable results of the studies mentioned. There is growing evidence that a source of transient *E. faecalis* may be food (Zehnder and Guggenheim, 2009). *E. faecalis* has been found a in wide range of processed and raw products especially dairy and meat products (Franz *et al*., 2003). Indeed, it has been used as a culture in cheese production (Giraffa, 2002). A study by Razavi *et al*. (2007) determined the clearance rate of *E. faecalis* following ingestion of a highly contaminated cheese. While it became clear that *E. faecalis* did not thrive in the oral cavity amongst the existing commensal microbiota, it took up to a week for the bacteria to be reduced to sub-detectable levels. Hence, the variable findings of *E. faecalis* in the oral cavity may be a

function of the dietary habits of the participants and the presence of transient bacteria originating from food.

On balance, it would seem that while *E. faecalis* can be found within the oral cavity it is probably not a commensal organism. This suggests the oral cavity does not generally favour this bacteria. However, this may change if an environmental change leads to an ecological niche developing which is suited to *E. faecalis* (Zehnder and Guggenheim, 2009). The high prevalence of *E. faecalis* in failed endodontically treated teeth suggests that root filled canals may provide such a niche if they become accessible via a carious lesion or leaking restoration. In such cases, transient *E. faecalis*, from the likes of food, may become established.

1.3.2 *E. faecalis* in Primary Root Canal Infections

E. faecalis is not considered a major pathogen in cases of primary endodontic disease, that is, endodontic disease that has not been previously treated. This is because it is rarely isolated from such canals.

Engström and Frostell (1964) found *E. faecalis* in 12% of primary root canal infections. Interestingly, this was not significantly different from the prevalence of isolates they obtained from root filled teeth. However, this lack of significance is not reflected in more recent studies, perhaps due to improved sampling techniques, taking greater care in preventing contamination and preserving the viability of organisms within samples. For example, Engström and Frostell's (1964) study used chloroform to remove some root fillings. Chloroform is now known to have some antimicrobial action and may have affected the prevalence or load of *E. faecalis* in persistent root canal infections (Molander *et al*., 1998).

The most cited microbiological study of primary root canal infections did not find any *E. faecalis* isolates, despite great care going into the culture and identification of all isolates present (Sundqvist, 1976). This finding was repeated in a high quality study by Sjögren *et al*. (1997). In contrast, using similar culturing techniques, Baumgartner and Falkler Jr, (1991) found *E. faecalis* to be

one of the most prevalent individual species in four out of the ten canals they sampled. This study, however, seems to be an outlier and given its small sample size and possible contamination issues at sampling could be questioned.

In contrast, molecular studies have shown a somewhat higher prevalence of *E. faecalis* in primary cases when compared to culture analysis. However, overall its occurrence still remains low. In a direct comparison of the techniques, Williams *et al.* (2006) found *E. faecalis* in 7% of canals with culture techniques compared with 13% for molecular methods. In a study specifically looking for *E. faecalis*, Siqueria Jr *et al.* (2002) only found a 7.5% prevalence out of 53 samples, while Fouad *et al.* (2002) found *Enterococcal* spp. in only 14% of primary cases they analyzed with PCR (Fouad *et al*., 2002, Siqueira *et al*., 2002). In contrast Rôças and Siqueira (2004) found a larger prevalence of 33% in primary endodontic infections. Nonetheless, when directly compared against the prevalence in root filled teeth within the same study, *E. faecalis* in primary infections was 50% less common.

In summary, *E. faecalis* has been reported to be present in primary root canal infections and within the oral cavity. However, on balance its prevalence and proportion is generally low relative to other bacteria. This is in contrast to its presence in persistent root canal infections.

1.3.3 *E. faecalis* in Persistent Root Canal Infections

E. faecalis is most well known in the endodontic literature for its association with cases of failed root canal therapy. Its prevalence in such cases is much higher than in primary endodontic infections or from the oral cavity (Molander *et al*., 1998, Sundqvist *et al*., 1998). What makes this even more significant is that in retreatment cases it has been shown that fewer species inhabit the root canal and by extrapolation the relative significance and proportion of *E. faecalis* in these cases is likely to be much greater than in primary infections (Molander *et al*., 1998, Sundqvist *et al*., 1998). Surprisingly, given the amount of research into endodontic microbiology over the past four decades, it has only been the past 15 years that much attention has been placed on the microbiota of failed root canal

therapy. The upside of this, however, is that much of the research is of a good methodological standard using both culture and molecular techniques.

As mentioned earlier, two of the most widely quoted studies on the microbiota of failed root canals were both published in 1998. Molander *et al.* (1998) investigated 100 root filled teeth with persistent apical periodontitis, 68% of these teeth sampled positive for intraradicular bacteria. *E. faecalis* was the most prevalent species being found in 47% of samples (Molander *et al*., 1998). The next highest prevalence was *Lactobacillus* spp. in 16% of cases. In 85% of canals only one or two species were recovered. Furthermore, 20 out of 32 cases that harboured *E. faecalis* were subjectively described as having heavy growth. These two findings both suggest a predominance of *E. faecalis* in both prevalence and proportion in persistent infections associated with previous root canal treatment.

Sundqvist *et al.* (1998), in the same year, published a study looking at the outcome of retreated teeth. They found 24 out of 54 teeth sampled positive for bacteria and of these nine (38%), contained *E. faecalis*. In all nine of these teeth *E. faecalis* was the only bacteria recovered. Again this study showed a prevalence and obvious predominance of *E. faecalis* bacteria in these teeth. This study went on to show that conventional disinfection protocols including irrigation with sodium hypochlorite and calcium hydroxide dressing only eliminated *E. faecalis* from 4 out of 9 canals from which it had been found prior to root filling. This persistence of *E. faecalis* resulted in a lower success rate than other canals without *E. faecalis*. Sundqvist's work therefore suggested *E. faecalis* is not only a highly prevalent and predominant species in failed root canal therapy but may also be responsible for the persistence of apical periodontal pathology after retreatment.

Hancock (2001) revealed similar results to Sundqvist *et al.* (1998) and Molander *et al.* (1998) in that *E. faecalis* was found in 30% of culture positive samples and in 66% of these it was the sole isolate from the respective canals. The significance of this work was that it was carried out in North America and thus

showed no difference in geographical location for *E. faecalis* prevalence when compared with the Scandinavian studies of Molander *et al*. (1998) and Sundqvist *et al*. (1998). Peculienne *et al*. (2001) found a much larger percentage of cases containing cultivable microbiota and a similarly larger prevalence of *E. faecalis* in 64% of positive sampled canals. *E. faecalis* was not as commonly a sole isolate in this study but was the dominant species in 19 out of 21 cases in which it was present.

Siqueira and Rôças (2004) used molecular techniques to find 77% of canals associated with failed endodontic treatment contained *E. faecalis*. This is the largest prevalence of any study although this was based on a small sample size of 22 teeth. Significantly, it was found most bacteria existed in mixed infections and thus questions whether the quantitative dominance of *E. faecalis* found in culture studies may be due to the limitations of culturing rather than the true state within these canals.

The preceding studies all indicate that *E. faecalis* is commonly found in persistent infections of root canal treated teeth. *E. faecalis* also seems to be quantitatively a much more dominant species in these cases than in primary root canal infections. Put together, these two findings suggest that *E. faecalis* may be an important pathogen in initiating or maintaining a post-treatment apical periodontitis. As a result, research into the efficacy of treatment procedures against *E. faecalis* is valid.

1.3.4 Why is *E. faecalis* so prevalent in root filled teeth?

As has been established, *E. faecalis* is much more prevalent in infections of root filled teeth than in necrotic untreated canals. There tend to be two hypotheses as to why this is. The first is that *E. faecalis* is capable of surviving initial endodontic treatment better than other bacteria and then re-establishing itself in the root filled tooth. Historically, the problem with this argument was that *E. faecalis* was rarely found in primary infections when culture techniques were used. However, this may have been because it was present in such low numbers as not to be detectable by culture methods (Sundqvist and Figdor, 2003). With the advent of

more sensitive molecular identification techniques *E. faecalis* is being detected in greater numbers from primary infections (Rôças and Siqueira, 2004, Williams *et al*., 2006). Hence, the possibility that *E. faecalis* may be surviving primary endodontic therapy and thus becoming the precursor for a persistent infection now seems more viable.

Nonetheless, for the above theory to hold true, *E. faecalis* still needs to have survived treatment. Sodium hypochlorite has been proven to be an effective antimicrobial irrigant against *E. faecalis* (Gomes *et al*., 2001). However, bacteria in deep recesses of the root canal system are protected from the actions of instrumentation and irrigation (Nair *et al*., 2005). A degree of resistance of *E. faecalis* to the most common medicament, calcium hydroxide, is a more established phenomenon and will be discussed further later (Haapasalo and Ørstavik, 1987, Ørstavik and Haapasalo, 1990). Given that this medicament is relied upon to diffuse into and eliminate microbiota within the deep recesses of the pulp-dentine complex, such a resistance is of concern. When *E. faecalis* has been found present in primary infections *in vivo,* antimicrobials have shown variable effectiveness at eliminating *E. faecalis*. (Williams *et al*., 2006, Sjögren *et al*., 1991). The possible persistence of *E. faecalis* in root canals after endodontic retreatment is a much greater possibility given its greater prevalence in such cases. However, no studies appear to have been done into the microbiology of failed retreatment cases.

The other hypothesis is that *E. faecalis* invades root canals secondary to endodontic therapy via leaking restorations or due to inadequate asepsis during treatment. Siren *et al.* (1997) has provided some evidence for this. Microbiological samples taken from 70 root canals that had been sent to a laboratory by general dentists were investigated - 34 cases had Enteric bacteria present and 36 did not. A questionnaire was sent to participating practitioners and asked for clinical information on the treatment of these teeth. It was found that in cases that had taken ten or more appointments *E. faecalis* was present in 12 out of 13 cases. So many appointments gives a much greater possibility of contamination during treatment with regular re-opening and the possibility of

rubber dam leakage. *E. faecalis* was also much more common in cases that at some stage had lost their seal perhaps due to a lost temporary filling (Siren *et al*., 1997). This study gives some evidence to the possibility of infection during or after treatment. However, infection during treatment still raises the concern over resistance to calcium hydroxide and other antimicrobials because once *E. faecalis* establishes itself in root canals undergoing treatment, it has been shown to be resistant to elimination by ongoing therapy (Chávez de Paz *et al*., 2003).

1.3.5 *E. faecalis* Virulence Factors

Virulence factors are coded by specific genes or combination of genes, which may or may not be present in all strains of *E. faecalis*. Not all have the same virulence factors because of the relatively high percentage of genes encoded on plasmids (Portenier *et al*., 2003). This indicates a relatively high degree of acquisition of certain proteins and factors that may enhance a strain's virulence as opposed to inherent virulence. However, there are a number of virulence factors commonly associated with *E. faecalis*.

1.3.5.1 Aggregation Substance

Aggregation substance is a plasmid-encoded bacterial adhesin. Sedgley *et al.* (2005b) found this gene encoded in all of the 33 Enterococcal endodontic isolates tested when genotypes were examined. It is a protein expressed by *E. faecalis* on its cell surface and is believed to be important in mediating contact with other bacteria, extracellular matrix and possibly also host cells (Portenier *et al*., 2003). Such an ability provides a number of benefits. An efficient contact between bacteria is important in aiding conjugation and transmission of other plasmids that may further enhance a bacteria's virulence (Kayaoglu and Ørstavik, 2004). Rozdzinski *et al.* (2001) has demonstrated an ability to bind extracellular matrix, including collagen type 1, twice as strongly in bacteria containing aggregation substance as those without. Such an ability may aid the formation of biofilms by allowing bacteria to act as early colonizers on a dentinal surfaces as well as to each other. This protein may also be involved in invasion of dentinal tubules (Love, 2001). Aggregation substance has also been implicated in

resistance to phagocytic killing of *E. faecalis* by binding to neutrophils and allowing phagocytosis independent of opsonisation. Paradoxically, phagocytosis by this method may impair subsequent intra-cellular killing due to perturbation in the subsequent phagosome (Rakita *et al*., 1999).

1.3.5.2 Surface Adhesins

Surface adhesins encompass a range of proteins encoded by various genes and are thought to be involved in attachment of bacteria to surfaces.

Surface protein Esp has been implicated in the ability of *E. faecalis* to form biofilms and attach to surfaces because Esp-defective bacteria were incapable of forming biofilms (Toledo-Arana *et al*., 2001). This protein has been found to be associated with pathogenic strains of *E. faecalis*, for instance those associated with endocarditis (Shankar *et al*., 1999).

Efa-A is a common gene expressed by *E. faecalis*, identified in 100% of samples taken from root canals by Sedgley *et al.* (2005b). It is very similar to a number of genes in *Streptococca*l spp. known to act as adhesins and thus is thought to have a similar function in *E. faecalis* (Kayaoglu and Ørstavik, 2004).

The *Ace* gene codes for a collagen binding protein thought to be involved in attachment of *E. faecalis* to extracellular matrix. Nallapareddy *et al.* (2000) has shown disruption of the gene disrupts binding to extracellular matrix. Hubble *et al.* (2003) and Kowalski *et al.* (2006) have both shown *Ace*-positive *E. faecalis* adhered to dentine much more effectively than *Ace*-negative strains probably because collagen makes up the organic component of dentine.

1.3.5.3 Gelatinase

Gelatinase is a proteolytic enzyme capable of direct and indirect damage to hosts via degradation of tissue (Portenier *et al*., 2003). It belongs to the same family of matrix metalloproteinases (MMP) that are produced by host cells and other bacterial cells involved in periodontitis. When unregulated release of gelatinase occurs from bacteria or in uncontrolled inflammation by host tissue, tissue

damage occurs (Kayaoglu and Ørstavik, 2004). This can be caused either directly by gelatinase or recruitment of host inflammatory cells capable of releasing more MMP and causing further damage and apical periodontitis. Such damage provides the bacteria with proteinacious nutrition. Gelatinase production is more common in *E. faecalis* than other species of *Enterococci* and has been found in 70% of endodontic isolates (Portenier *et al*., 2003, Sedgley *et al*., 2005b).

1.3.5.4 Cytolysin

Cytolysin is another plasmid encoded protein but has also been reported to be incorporated into the chromosome (Ike and Clewell, 1992). It is capable of lysing a number of host cells including neutrophils and macrophages and as a result interfering in host inflammatory and immune response (Kayaoglu and Ørstavik, 2004). Such a virulence factor helps in preventing phagocytosis and aids the establishment and proliferation of bacteria. Furthermore, cytolysin has also demonstrated bacteriocin activity against a range of Gram positive bacteria (Jett and Gilmore, 1990). Given that *E. faecalis* is commonly found in persistent infections of endodontically treated canals often as a sole isolate or with low numbers of other species, it may be that *E. faecalis* reduces competition in the root canal by producing antimicrobials like cytolysin. Strains of *E. faecalis* positive for cytolysin have also been shown to have a greater aggregation response than those without. A review by Kayaoglu and Ørsatvik (2004) stated that the prevalence of the cytolysin gene is variably reported in 16-60% of isolated strains.

1.3.5.5 Lipoteichoic Acid

Lipoteichoic acids (LTA) are a group of acids commonly found on cell surfaces of many Gram positive bacteria (Wicken and Knox, 1975). They are interesting as virulence factors because they appear to contribute to many virulent traits. LTA from other species have shown an affinity for hydroxyapatite and thus adhesion to dental hard surfaces (Ciardi *et al*., 1977). Such an affinity may help early colonization and the establishment of biofilms within the root canal. LTA from strains of *E. faecalis* can stimulate the leukocyte release of several inflammatory mediators (Kayaoglu and Ørstavik, 2004). Such mediators may provide protein

nutrient for the bacteria, cause potential tissue damage and may be involved in apical periodontitis development. Most interesting is the possible role LTA plays in resistance to adverse conditions and possibly to root canal medicaments. Signoretto *et al.* (2000) reported a doubling in quantity of LTA produced when *E. faecalis* was in the 'so-called' viable but non-cultivable (VBNC) state. Thus LTA presents an array of potential virulence traits that not only enhance pathogenicity but also its resistance to host and introduced anti-microbial factors.

Research into the virulence factors associated with *E. faecalis* is ongoing. However, it is clear that this bacteria contains a diverse array of factors that may contribute to its survival in the root canal system. Furthermore, as with cytolysin, some of these virulence factors may be up or down regulated as environmental conditions change and thus exhibit a potentially adaptive phenotype (Appelbe and Sedgley, 2007). Such adaptation of virulence factors may not only make this bacteria capable of establishing an endodontic infection but also provide mechanisms for resistance to elimination procedures.

1.4 Biofilm

Biofilms are a natural form of microbial growth. They were first described by in the 1970s and since this time their definition has evolved (Costerton *et al.*, 1978). Today, the most common definition is that of a community of microbiota attached to a surface or each other and embedded in extracellular matrix (Hall-Stoodley *et al.*, 2004). In natural environments bacteria have such a strong tendency to become associated with surfaces that the vast majority of bacteria in nature grow and function as biofilms (Costerton *et al.*, 1995, Svensåter and Bergenholtz, 2004). Biofilms are ubiquitous and recalcitrant, posing problems in medical, industrial and environmental microbiology. They are currently thought to be the cause of over 50% of human infections and are notoriously difficult to eradicate (Costerton *et al.*, 1999).

Basic biofilm development and community structure is universal (Donlan and Costerton, 2002, Svensåter and Bergenholtz, 2004). A conditioning film of macromolecules from the environment adsorb to a surface before early bacterial colonizers attach to that surface and each other. Growth occurs with division of constituent cells and attachment of late colonizers as the micro-environment of the biofilm changes to accommodate them. Extracellular matrix is produced by the microbiota and can include polysaccharides, proteins and even nucleic acids. Regular detachment of bacteria is a normal aspect of biofilm development whether it is due to physical dislodgement or even an organized cleavage controlled by the biofilm population itself (Svensåter and Bergenholtz, 2004). Bacteria themselves constitute approximately 15% by volume of biofilm mass while the extracellular matrix constitutes 85% (Donlan and Costerton, 2002). Microcolonies are the basic building block of biofilms. Water channels intersperse and delineate these microcolonies which are of a heterogeneous nature throughout the biofilm (Svensåter and Bergenholtz, 2004). Such heterogeneity occurs through the development of gradients of nutrients, waste products and other physicochemical factors such as oxygen tension that develop throughout a biofilm. Different micro-environments favour different species of

bacteria and different phenotypes of the same bacteria that subsequently arrange themselves according to these ecologically driven determinants (Chávez de Paz, 2007). As a result, biofilm communities are dynamic, capable of changing and evolving as conditions change (Donlan and Costerton, 2002). In the endodontic context, such an environmental change may be caused by treatment with anti-microbial agents.

From an evolutionary perspective, the formation of biofilms must logically confer a survival advantage to those colonizing bacteria. Biofilms are robust entities and are notoriously difficult to remove and destroy, hence their regular association with persistent infections (Costerton *et al.*, 1999). The development of different micro-environments as biofilms evolve may create favourable conditions for different species to join and colonize. For example the metabolic by-product of one species may be a primary nutrient for another. Different species may also contain different proteinases capable of breaking down components of macromolecules for metabolism. By organizing a collective physiology within a biofilm, different bacteria may work synergistically to optimally breakdown larger molecules and thus access more nutrition than they would be capable of as individual planktonic bacteria (Marsh and Bowden, 2000). Such species may thus find the biofilm conditions much more favourable than the external environment. This would allow them to thrive when previously they may have only been present in small numbers planktonically.

Biofilms may also enhance individual bacterial pathogenicity by providing the possibility of easier microbial interactions between synergistic species (Svensåter and Bergenholtz, 2004). Many examples of microbial interaction within the root canal space exist. The classic example is that from the Fabricius *et al.* (1982) monkey study. When inoculated as a mono-species into devitalized monkey root canals, *Bacteroides oralis* could not be recovered six months later. However, when inoculated in equal combination with seven other bacteria, *B. oralis* became the dominant species. Given that the environments were the same, such a difference in survival was hypothesised to be linked to microbial interactions. Given our current knowledge of biofilm and endodontic

microbiology it would seem most logical that such interactions occurred in the biofilm context.

1.4.1 Biofilm Mechanisms of Antimicrobial Resistance

The most significant advantage that biofilms provide from an endodontic treatment standpoint, is an increased resistance to both antimicrobial agents and the host defence mechanisms. Studies have shown that when bacteria are embedded within a biofilm they can be up to 1000 times more resistant to some antimicrobials compared to their planktonic state (Stewart and Costerton, 2001). A number of potential mechanisms have been suggested.

1.4.1.1 Diffusion Barrier

In order for an antimicrobial agent to affect a bacterial cell it must first come into the proximity of that cell. Biofilms can form physical barriers to the diffusion of antimicrobial agents into the biofilm structure (Costerton *et al*., 1999). However, not all agents are physically prevented from penetrating a biofilm to the same degree (Stewart, 1996). For example, a physical barrier would not cause a major problem for small molecules such as hydroxyl ions from calcium hydroxide medication in root canals.

A more significant hindrance to the diffusion of antimicrobial agents through biofilms may be interactions between the agents and the constituents of the biofilm (Fux *et al*., 2005). Once the surface layer of a bacterial biofilm is affected by an agent this does not necessarily mean the underlying layers will be immediately accessible. This is because the extracellular material and even remnants of dead cells will be retained and can still act as a diffusion barrier. Non-specific antimicrobials such as the hydroxyl ion of calcium hydroxide may interact with both live and dead bacteria and extracellular material resulting in it being depleted and reducing its efficiency. Another example of an interaction is beta-lactamase. It is believed to accumulate in the extra-cellular matrix where it may deactivate beta-lactam antibiotics. Beta-lactamase producing bacteria within biofilms have been shown to have greater resistance to ampicillin than

their non-beta-lactamase producing counterparts (Anderl *et al.*, 2000). Negatively charged polymers in the matrix may also retard the progression of positively charged antimicrobials. This is thought to be the case for aminoglycoside antibiotics and could conceivably be the case for positively charged endodontic agents such as chlorhexidine (Abdullah *et al.*, 2005, Fux *et al.*, 2005).

The result of such barriers to diffusion means the penetration of antimicrobials will be slowed to some extent and may conceivably give bacteria within the biofilm greater time to adapt to changes and potentially increase their chances of survival (Fux *et al.*, 2005).

1.4.1.2 Antimicrobial Deactivation

As previously noted, biofilms are heterogeneous and capable of establishing a range of microenvironments within their multi-cellular biomass (Distel *et al.*, 2002, Stewart and Costerton, 2001). For example, it has been shown that oxygen is rapidly used up at the surface resulting in a more anaerobic environment in deeper layers (De Beer *et al.*, 1994) In such an environment aminoglycoside antibiotics are much less effective (Stewart and Costerton, 2001). pH gradients may also be established if acidic waste products are not efficiently removed. As a result, an alkaline medicament like calcium hydroxide may be less effective.

1.4.1.3 Slow rate of Bacterial Growth

Despite the limits imposed by physical and physiological barriers within a biofilm, antimicrobial agents may still reach target organisms. However, bacteria within biofilm grow significantly more slowly than their planktonic counterparts (Donlan and Costerton, 2002, Fux *et al.*, 2005). This may be due to phenotypic changes induced by attachment, communication with neighbouring cells, build-up of metabolic waste, nutrient limitation or increased energy requirements for other biofilm processes (Stewart and Costerton, 2001). Regardless, a slow growing or slow dividing state can be considered similar to planktonic cells in the stationary phase. This would become a problem for an antibiotic such as penicillin which acts against cell wall synthesis and thus requires a bacteria to be

growing to exert its effect (Tuomanen *et al*., 1986). *E. faecalis*, in a stationary-like phase, has been shown to be resistant to killing by calcium hydroxide (Portenier *et al*., 2005).

1.4.1.4 Expression of Resistant Phenotypes

A change in the rate of growth of bacteria is not the only phenotypic change related to biofilm formation. It has been demonstrated that gene expression by cells can vary as much as 35-50% between biofilms and their planktonic counterparts (Sauer *et al*., 2002). Some phenotypic changes such as the activation of adhesive-type genes, communication genes and matrix producing genes are simply necessary to form a biofilm (Fux *et al*., 2005). These changes are not completely understood but given the increased resistance of biofilm over planktonic bacteria it would not be surprising if some of these phenotypic changes directly contribute to the resistance of individual bacteria within biofilms.

A developing theory is that a few bacterial cells within a biofilm develop a biofilm-specific phenotype (Stewart and Costerton, 2001, Fux *et al*., 2005). This theory comes as a result of an increased resistance of bacteria within very thin, young biofilms that cannot alone be explained by the other mechanisms of resistance already mentioned (Das *et al*., 1998). Other studies have also shown the survival of small populations of cells within biofilms even when 99% of the population have been killed by an antimicrobial challenge. The term persister cells has been coined for these survivors and they are thought to tolerate antimicrobial challenges by entering a spore-like state in which they do not divide and grow but remain viable while under stress (Lewis, 2007). They are commonly thought to occur in biofilms and possibly are a key factor in the recalcitrant nature of biofilm infections (Lewis, 2007).

1.4.1.5 Quorum Sensing

The phenotypic alterations of bacteria within biofilms is predominantly ecologically driven (Chávez de Paz, 2007). However, in biofilms due to the close proximity of neighbouring bacteria it is likely that a degree of communication is

established via signaling molecules that can pass between bacteria and mediate phenotypic changes such as virulence, biofilm development, adaptive responses to stress and entry into the stationary phase (Cvitkovitch *et al*., 2003). This phenomenon is known as quorum sensing and may allow populations of bacteria to act in a complimentary fashion.

1.4.1.6 Genetic Transfer

The transfer of plasmids via conjugation is a well known method of transfer of genetic material between bacteria of the same and different species. Such plasmids may encode for molecules that can enhance virulence and may provide means of antimicrobial resistance. This method of transfer requires bacterial cells to be in close proximity. Conjugation is not restricted to bacteria within biofilms and thus is not strictly a biofilm related mechanism for enhanced survival. However, given the dense arrangements of bacteria within biofilms, biofilms are an ideal state in which genetic transfer is likely to occur (Fux *et al*., 2005). Sedgley *et al*. (2008) has demonstrated such transfer of plasmids between *E. faecalis* and *S. gordonii* when combined *ex vivo* in prepared root canals.

1.4.2 Evidence of Biofilms in Endodontology

Nair (1987) first described the presence of biofilm-like structures in root canals. He studied 31 untreated, non-vital teeth with apical periodontitis using transmission electron microscopy. He described for the first time the presence of dense aggregates of single or multiple species sticking to the dentinal walls. They were arranged in either single or multi-layers and appeared to be suspended in a loose amorphous material. Although the term biofilm was not used, the resemblance to dental plaque, which is the most well-known dental biofilm, was noted.

Molven *et al.* (1991) followed up Nair's work by using scanning electron microscopy (SEM) to view several roots of extracted non-vital teeth with apical periodontitis. SEM allowed the topography of the microbiota inside the root to be viewed. He observed several morphological types of bacteria including rods,

cocci and spirochaetes. Aggregates of cocci and rods were observed in what were described as micro-colonies including a corn-cob arrangement of cocci attached along the length of rods.

Subsequent studies seemed to focus on the presence of extra-radicular biofilms with repeated evidence for such (Leonardo *et al*., 2002, Tronstad *et al*., 1990). Bacterial forms were seen within an amorphous material extending out from the apical foramen and included cocci, bacilli and filaments. It was suggested that these biofilms were formed and originated outside the root canal on the external root. Another possible explanation is that these structures are actually external extensions of intra-radicular biofilms. In this case they may not actually be attached to the external root surface and only appear to be so as a result of SEM processing. The latter would seem the more likely explanation given the very high success rates of non-surgical root canal treatment.

A more recent SEM investigation into the intra-radicular presence of biofilms was carried out by Siqueria (2002). It was similar in methodology to that of Molven *et al.* (1991), as were the results. Intra-radicular bacteria were observed arranged in a biofilm-like appearance involving both single and multi-species aggregations. Most areas of the observed root canals had dense aggregations of bacteria attached to dentine walls and in some cases showed tubule invasion. The colonisation of the walls was not uniform with much variation in the species attachment and in the predominance of certain species within the larger aggregates. Most areas had a dense colonization but in some areas it was more sparse (Siqueira, 2002).

Nair *et al.* (2005) again discussed the presence of biofilms when they looked at the bacteriological results of single-visit endodontic treatment on apical root sections. After treatment with instrumentation, irrigation and obturation in one visit, mesial roots of lower molars were surgically exposed and the apical 2mm resected. They were then examined with TEM. In 14 of 16 teeth treated in one-visit appointments, bacteria were found growing as biofilms in inaccessible recesses of the root canals such as isthmuses and accessory canals of the roots.

The biofilms consisted of mixed microbial infections held together with an extracellular matrix substance attached to adjacent dentinal walls. Unfortunately there was no control group in this study that looked at the effect of calcium hydroxide medication on these biofilms.

A unique light microscopic study by Ricucci and Siqueria (2010) has recently produced some of the most compelling evidence so far for the presence of biofilms in root canals. From 100 teeth including both untreated and treated canals, 106 roots were sectioned and stained. Intra-radicular bacteria were found in 99% of cases and biofilms identified in 77% of cases. The biofilms were normally multi-layered and consisted of a number of bacterial morphotypes although one morphotype normally dominated. The bacteria were embedded in extracellular matrix although the ratio of matrix to bacteria varied greatly between different biofilms. Some of the images of biofilm attached to dentinal wall are amongst the most convincing evidence yet of the existence of biofilms in infected root canals.

Together these studies provide compelling evidence that biofilms are the major state in which bacteria within infected root canals grow, develop and evolve. Such a growth phase would be in keeping with most bacteria found in other natural environments. The biofilm state provides bacteria with a range of physical and adaptive mechanisms with which to survive within the root canal and resist the antimicrobial insults of endodontic treatment.

1.5 Calcium Hydroxide

Calcium hydroxide was first recommended for use in endodontics in the 1920's (Siqueira and Lopes, 1999). Since then it has become the most widely used medicament in endodontics for inter-appointment dressings. Calcium hydroxide is a base with a pH of approximately 12.5. It has a low solubility in water and so when applied as an endodontic paste it does not dissolve and diffuse as readily as more soluble alkali compounds such as sodium or potassium hydroxide, making it a safer compound for periradicular tissues. In aqueous solution calcium hydroxide dissociates into calcium and hydroxyl ions. The antimicrobial effects of calcium hydroxide are exerted by hydroxyl ions (Siqueira and Lopes, 1999).

A number of favourable properties have been attributed to calcium hydroxide when used as an endodontic medicament including an antimicrobial action, tissue dissolving capability, an inhibition of inflammatory root resorption and the induction of an apical hard tissue barrier (Siqueira and Lopes, 1999). It is the antimicrobial effect of calcium hydroxide that is by far its most utilised property. Following mechanical debridement and irrigation 50-60% of infected root canals are rendered bacteria free (Byström and Sundqvist, 1983, McGurkin-Smith *et al*., 2005, Shuping *et al*., 2000). However, the problem for the clinician is that it is not possible to tell which of these canals are and which are not still infected. Therefore, calcium hydroxide has been placed empirically as an inter-appointment dressing and been shown to result in a significantly greater percentage of canals sampling free of cultivable microorganisms (Byström *et al*., 1985, Shuping *et al*., 2000).

The mechanism or targets against which calcium hydroxide exerts this antimicrobial effect are not entirely known but it is a non-specific antimicrobial and as a result probably involves a number of different pathways of damage. Siqueira and Lopes (1999) have suggested three main mechanisms of action :

1. Cytoplasmic membrane damage: the cytoplasmic membrane is crucial to the survival of any cell type. Hydroxyl ions are thought to result in lipid peroxidation and consequent damage to the lipids that make up the membrane. This damage can result in unregulated membrane permeability and dysfunction of many cellular processes that are dependant on functional cell membrane and the proteins embedded within it.

2. Protein denaturation: Homeostasis in the cellular environment produces a very narrow biological pH range. When the pH increases, excess hydroxyl ions can interact with the ionic bonds that control the folding or tertiary structure of proteins. Variations in the physical structure of these proteins can result in a loss of their biological activity.

3. DNA damage: hydroxyl ions are capable of splitting DNA resulting in damage to chromosomes, loss of genes and deregulation of cellular activity.

Despite its high pH and multiple mechanisms of action, it is well recognised that calcium hydroxide is not without limitations when eliminating bacteria from the root canal system. These may be due to its physical properties, host or bacterial factors. Calcium hydroxide has even been implicated with a potentially harmful risk of selecting for *E. faecalis* which may result in a more resistant and difficult infection to retreat (Chávez de Paz *et al*., 2003). Whether or not application of calcium hydroxide improves success is currently an important topical subject in endodontics. However, if it were that calcium hydroxide was selecting for a more resistant persistent infection, then its use would be a much more serious question and could violate the medical principle of doing no harm.

1.5.1 Efficacy of Calcium Hydroxide

As previously mentioned, it is well accepted that instrumentation and irrigation is not completely effective at eliminating bacteria from all canals undergoing endodontic therapy (Byström and Sundqvist, 1983, McGurkin-Smith *et al*., 2005, Shuping *et al*., 2000). Unless microbial sampling is carried out it cannot be known which canals still contain residual bacteria and it has been shown that such canals are more likely to fail (Sjögren *et al*., 1997). Furthermore, microbial sampling in itself is not a guarantee that a root canal is indeed sterile. The addition of calcium hydroxide empirically as an intracanal medicament between appointments has been shown *in vivo* to result in a further 20-30 % reduction in bacteria-free canals (Byström *et al*., 1985, Law and Messer, 2004, Shuping *et al*., 2000). However, recent outcome studies comparing single and multi-visit endodontic treatment have not been able to show a significant difference (Peters and Wesselink, 2002, Sathorn *et al.,* 2005, Trope *et al*., 1999, Weiger *et al*., 2000). The fundamental difference between single-visit and multi-visit treatment in these studies was the use or not of calcium hydroxide as an inter-appointment dressing. Therefore, interest has focused on the efficacy of calcium hydroxide as an antibacterial medicament. Conflicting results as to the effectiveness of calcium hydroxide have been regularly published since the 1980s. Overall there seems to be a trend towards *in-vivo* studies favouring calcium hydroxide while *in vitro* studies seem to highlight some deficiencies. However, some more recent *in vivo* studies also seem to show a lack of efficacy of calcium hydroxide and thus deserve some further attention.

In vivo studies have historically shown calcium hydroxide to be an effective antibacterial intracanal medicament. Byström and Sundqvist (1985) demonstrated this when they applied a calcium hydroxide dressing into 35 infected canals for four weeks. After the dressing was removed the canal was left empty for 2-4 days to allow for any possible rebound of residual surviving bacteria. After this period a further sample was taken. Bacteria were detected in

only one of the 35 canals. Thus, calcium hydroxide rendered 97% of canals bacteria free, within the limitations of culturing.

A subsequent study by Sjögren *et al.* (1991) looked at the length of time required for calcium hydroxide to exert its action. They took 18 teeth which were instrumented with an ultrasonic technique and irrigated with 0.5% sodium hypochlorite. After this initial treatment nine of the 18 teeth tested positive for bacteria. After application of calcium hydroxide for one week, no bacteria were detected in any samples.

In the late 1990's Shuping *et al.* (2000) carried out a study aimed at replicating that of Byström *et al.* (1985) except that rotary instrumentation was used. Once again, instrumentation and irrigation rendered 63% of canals bacteria free. A further calcium hydroxide dressing placed for a minimum of one week resulted in 93% of canals sampling negative for bacteria. Overall, these studies show a favorable antibacterial efficacy for calcium hydroxide when tested *in vivo*.

In contrast, over the same period of time a number of *in vitro* studies found that calcium hydroxide may not necessarily be capable of eliminating all bacteria from the root canal system. In response to work by Akpata and Blechman (1982) showing the invasion of dentinal tubules by root canal bacteria, Haapasalo and Ørstavik (1987) developed an *in vitro* model that achieved dentinal invasion. Using bovine dentine specimens and suspending them in *E. faecalis* broth for three weeks, SEM and light microscopy demonstrated tubule invasion. Dressings of calcium hydroxide and camphorated paramonochlorophenol (CMCP) were both tested. Within one day of CMCP treatment no detectable bacteria were found whereas after ten days with calcium hydroxide *E. faecalis* was still detected. One particularly important difference between this study and the *in vivo* studies already discussed was how the samples were taken. With *in vivo* studies, samples were taken with paper points and may only have sampled bacteria from the main root canal or possibly from the portion of dentine tubules very close to the main canal. However, Haapasalo and Ørstavik (1987) used progressively larger burs to remove and collect dentine dust and chips as it was

removed from the main canal towards the root periphery. This method showed survival of bacteria deep within dentinal tubules. Over time, survival of bacteria in the main root canal and inner dentine decreased while bacteria in the outer dentine survived. This demonstrated a degree of protection from medicaments the further bacteria were from the main canal.

Ørsatvik and Haapasalo (1990) repeated their experiment using a range of other medicaments and different bacteria. Again after ten days application of calcium hydroxide, *E. faecalis* was unable to be eliminated. It was more effective against the other bacteria tested, namely *Streptococcus sanguinis, Escheria coli* and *Pseudomonas aeruginosa* although less effective than CMCP.

While Safavi *et al*. (1990) used the same model for tubule infection they did not sample for the presence of bacteria by removing dentine. Instead they simply placed their dentine specimens in broth and observed the development of any turbidity. Iodine solution (IKI) and calcium hydroxide were the test antimicrobials and samples were exposed for 24 hrs. Using their detection method, IKI was more rapidly effective than calcium hydroxide. This is a commonly quoted paper in regard to the relative ineffectiveness of calcium hydroxide but seems to lack clinical relevance as the medicament was only placed for 24 hours.

Like Ørsatvik and Haapasalo (1990), Siqueira and Uzeda (1996) wanted to test this model against bacteria other than just the pH resistant *E. faecalis*. Thus, *Actinomyces israeli*, *E. faecalis* and F*usobacterium nucleatum* were tested. Once again calcium hydroxide proved unable to eliminate *E. faecalis* from this model even after one week exposure. However, it was also not able to kill *F. nucleatum* although *A.israelii* was eliminated after one hour exposure (Siqueira and Uzeda, 1996).

Given that chlorhexidine has been given greater consideration as an endodontic antimicrobial in the last decade, Schafer and Bossmann (2005) and Gomes *et al.* (2003) in different studies tested it using similar models to Haapasalo and

Ørstavik (1987). Chlorhexidine was found to be significantly more effective than calcium hydroxide at eliminating bacteria over a range of time periods.

Together, these *in vitro* studies seem to suggest a relative lack of efficacy of calcium hydroxide compared with other medicaments. Certainly they do not confirm the findings of the aforementioned *in vivo* studies. A number of issues with regard to methodology need to be considered and appreciated. The experiments of Safavi *et al*. (1990) did not sequentially test for viable bacteria deep within dentine. Therefore this test was not as thorough as Haapasalo and Ørsatvik's (1987) study and may give a false impression of the antimicrobial's abilities because diffusion capabilities were not necessarily tested. All studies except for Schafer and Bossmann (2005) also used bovine dentine. While Haapasalo and Orstavik (1987) claimed that such dentine is similar to humans, bovine dentine has larger tubules and may have had an effect on tubule invasion and thus medicament efficacy.

The *in vivo* studies noted also have their limitations. In particular, the efficacy of microbial sampling has been called into question. Such sampling was carried out with paper points using specially formulated sampling medium. These techniques were originally developed by Möller (1966) in order to prevent contamination, reduce the influence of residual antibacterial agents and collect as many residual bacteria as possible. However, it cannot be definitively determined if all residual medicament has been neutralized and that all oxygen sensitive bacteria have been protected. Even more significantly, paper points are unlikely to be collecting residual bacteria deep within tubules and accessory canals. The significance of these bacteria in terms of producing a sterile pulp-dentine complex has been demonstrated in the *in vitro* studies discussed. How significant tubule infection is clinically to a successful treatment outcome is another matter that is yet to be been determined. Nonetheless, if producing a sterile pulp-dentine complex is the goal of endodontic treatment, such tubule invasion needs to be considered and dealt with.

As well as an appreciation of the limitations of individual studies it is important to appreciate the limitations of the comparison between these *in vitro* studies with the *in vivo* ones. It is very difficult to determine the clinical relevance of *in vitro* studies but the aim of these studies should always be to simulate as well as possible the clinical situation. However, despite regular citations used to point out the limitation of calcium hydroxide as an endodontic medicament, the preceding *in vitro* studies use a very different quality of infection to the *in vivo* studies discussed. The *in vivo* investigations discussed were all primary endodontic infections, likely to comprise polymicrobial, Gram negative, obligately anaerobic bacteria. As discussed earlier (section 1.3.2), very few would contain *E. faecalis*. However, the *in vitro* studies mostly involved mono-species infections of *E. faecalis*. Thus, to question the clinical efficacy of calcium hydroxide in the initial treatment of a primary infection on the basis of *in vitro* studies utilizing very different infection models seems a tenuous argument. A more relevant argument might be that calcium hydroxide may not be an effective medicament in endodontic retreatment cases where *E. faecalis* is a more prevalent species and may more often be found as a dominant species in such persistent infections. In this sense very few quality *in vivo* studies exist with which to make a valid comparison. However, one such study carried out by Sundqvist *et al.* (1998) did concur somewhat with the findings of the *in vitro* studies in that three out of the nine canals in which *E. faecalis* was originally isolated still sampled positive for viable *E. faecalis* after endodontic chemo-mechanical preparation including a calcium hydroxide dressing. This is certainly not as dire as the complete ineffectiveness of calcium hydroxide as suggested by some *in vitro* studies but it does show conventional root canal therapy, including calcium hydroxide dressing, to be less effective in retreatment cases than what is reported in *in vivo* studies of primary infections.

The *in vivo* studies already discussed were all completed prior to 2000 but are still among the most widely cited and well conducted studies on the microbiological effect of endodontic treatment. However, in the last ten years a number of *in vivo* studies have been conducted looking at the same effect. Despite the fact that essentially the same sampling techniques for microbial

culturing were used as in the earlier *in vivo* studies, results of the newer studies are quite different. These studies have increased the question marks over calcium hydroxide dressing even further than some of the *in vitro* studies already discussed because they seem to show a lack of calcium hydroxide efficacy in an obviously clinically relevant situation and against infections that are generally thought to be less resistant than those dominated by *E. faecalis*. The first major *in vivo* study to cast doubt on calcium hydroxide as an effective antibacterial medicament was by Peters *et al*. in 2002. Initially after instrumentation and irrigation with a 2% sodium hypochlorite solution, 85% of canals tested were negative for bacteria. Surprisingly after a four week application of calcium hydroxide in the same canals this number had actually dropped to 28%. In other words, despite the presence of calcium hydroxide in the canals, the implication was that bacteria had actually been able to grow and recover to detectable levels (Peters *et al*., 2002). This is even more surprising given the infections were polymicrobial and not restricted to more resistant bacteria such as *E. faecalis*. Since this study gave such contradictory results to previous studies without apparent significant differences in methodology it has been viewed with some skepticism and justifiably been scrutinized for possible bias and errors. Potential sources of error could include the effectiveness of calcium hydroxide application into the root canal and whether contamination occurred between appointments. In contrast to other studies and somewhat counter-intuitively this study of Peters *et al.* (2002) also resulted in greater clinical and radiographic healing when bacteria were present in canals immediately prior to obturation than if the canals tested negative (Peters and Wesselink, 2002, Sjögren *et al*., 1997, Sundqvist *et al*., 1998).

However, the work of Peters *et al.* (2002) was soon followed by a study from Waltimo *et al*. (2005) that reported a similar, although not as drastic, increase in the number of canals that tested positive for bacteria after calcium hydroxide dressing compared with before. Prior to dressing 22% tested positive and after dressing 33% tested positive. Once again calcium hydroxide proved either at best ineffective and at worst seemed to support bacterial growth. Furthermore, similarly to the study of Peters *et al.* (2002), the bacterial flora taken from these

canals did not contain high percentages of bacteria that are known to be more resistant to calcium hydroxide such as *E. faecalis*. Even if it is accepted that calcium hydroxide may not be effective in eliminating bacteria, another of its functions is to temporarily fill the root canal, removing space for bacteria to grow and preventing access to nutrition. It would seem that in these studies this function is not occurring either. The concept that calcium hydroxide may be ineffective is not new, but the fact that these studies tend to show it supporting growth is more controversial and difficult to rationalize.

Not only has it been suggested that calcium hydroxide is ineffective by these new *in vivo* studies, some newer studies are also demonstrating the superiority of other medicaments. For instance, Chu *et al*. (2006) found no difference between calcium hydroxide, Ledermix and Septomixin. The latter two are antibiotic medicaments, which are generally considered to be less effective than antiseptic medicaments. Between the three dressings only 52-69% of canals were rendered bacteria free, which is in the range of what would be expected by instrumentation and irrigation alone without a one-week intra-canal dressing. A less controlled study by Chávez de Paz *et al.* (2003) analyzing sampling results from a broad range of practitioners revealed that IKI was more effective as an inter-appointment medicament than calcium hydroxide and that calcium hydroxide dressing was more commonly associated with the presence of *Enterococcus* species post medication.

With this growing realization that eliminating the endodontic infection in its entirety is an unrealistic goal, attention must be drawn to the changes in the root canal microbiota caused by endodontic treatment. Some studies have discussed the concept that the use of calcium hydroxide may select for more resistant bacteria (Chávez de Paz *et al*., 2007, Hancock, 2001, Sundqvist, 1992). This may occur by selection of bacteria innately resistant to high pH or, of more concern, calcium hydroxide may initiate stress mechanisms or adaptive responses that increase the overall resistance of any persistent infection. Such selection could potentially lead to a much more recalcitrant and difficult to treat infection. Waltimo *et al*. (2005) have reported an interesting appraisal of the allogenic

changes caused by conventional endodontic treatment. Instrumentation and irrigation gave a generalized decrease in the microbial flora without favouring any particular species. In contrast, the post medication samples showed a distinct change in relative proportions of the microbiota present. Medication with calcium hydroxide favoured Gram positive facultative bacteria (Waltimo *et al*., 2005). A similar trend is seen in a study by Kvist *et al*. (2004).

E. faecalis is a Gram positive, facultative bacteria and would seem a prime example of a bacterial species which such selective processes may favour. Indeed, Sundqvist *et al.* (1998) found this bacteria resisted endodontic therapy, including calcium hydroxide, better than other bacteria *in vivo*. It would seem fair to conclude from the preceding discussion that calcium hydroxide has a questionable efficacy especially against *E. faecalis*. A degree of resistance to calcium hydroxide may be due to virulence factors that provide both inherent resistance and adaptive mechanisms to cope with high pH challenges. Given that a residual infection is likely to remain following root canal treatment due to the complexity of the root canal system, some bacteria are more likely to be favoured than others. pH tolerant bacteria such as *E. faecalis* are likely candidates for such selection given their greater resistance to calcium hydroxide dressing. Thus, it may be concluded that calcium hydroxide might be selecting for a particularly resilient, well-adapted infection that could be more resistant to endodontic treatment procedures than the initial primary infection. Such resistance may be one of several contributing factors to the somewhat reduced success rates of endodontic retreatment procedures against persistent endodontic infections (Sjögren *et al*., 1990).

1.5.2 Challenges to Calcium Hydroxide

1.5.2.1 Dentinal Tubules

The term pulp-dentine complex refers to the communication between the pulp and dentine by way of patent dentinal tubules resulting in a continuous system. Odontoblast cell bodies line the main root canal in healthy pulp and have processes that extend into the dentinal tubules. Radicular dentinal tubules extend from the pulp to the cementum, narrowing in diameter as they do so. This combined with the fact that tubules diverge as they move outwards means they are much more densely packed together in inner dentine than outer dentine (Garberoglio and Brännström, 1976). There are also regional variations in the dentinal tubule concentration with them being much more concentrated coronally than apically with 42,360 tubules/mm^2 in cervical root dentine vs 8,190 tubules/mm^2 in apical root dentine (Carrigan *et al*., 1984).

Love (1996) has demonstrated invasion by *Streptococcus gordonnii* and *E. faecalis* into human dentinal tubules. Of note was the regional variation in infection with much greater bacterial penetration occurring coronally and in the mid-root than apically. This was accorded to the variation in concentration of tubules throughout the root as mentioned above. The depth of tubule penetration from the root canal has also demonstrated to be variable but Peters *et al.* (2001) showed bacteria to be present in the outer third of dentine in 62% of samples from infected human teeth. In order to disinfect the pulp-dentine complex calcium hydroxide must diffuse in sufficient concentration through dentinal tubules.

1.5.2.2 Diffusion

Endodontic medicaments cannot always be delivered directly into dentinal tubules and must usually rely entirely on diffusion of the medicament's active component into and through the dentine to eliminate bacteria. However, studies have shown calcium hydroxide is unable to completely disinfect dentinal tubules even under ideal *in vitro* conditions presumably due to an inability to diffuse

sufficiently to reach a lethal concentration (Ørstavik and Haapasalo, 1990, Safavi *et al*., 1990).

In terms of the general principles of diffusion, a gradient of ion concentration from the inner pulpal dentine to the outer dentine drives the diffusion equation. Nerwich *et al.* (1993) demonstrated a diffusion gradient of hydroxyl ions through the tooth root following the application of calcium hydroxide by measuring pH. They also showed that calcium hydroxide diffused less readily in apical dentine compared to cervical dentine as the maximum pH was less. This observation neatly exhibits the influence of anatomy on the action of medicaments. Apical variation in diffusion was explained by the decreased number of tubules and their decreased diameter apically (Nerwich *et al*., 1993, Carrigan *et al*., 1984).

Pashley (1985) has investigated the permeability and diffusion of a range of substances through dentine and the physical properties of dentine that affect this. The most significant properties are the diameter of the dentinal tubules and their density (Pashley, 1985). A large portion of any tubule may be taken up by intra-tubular material thus reducing its diffusion. Furthermore, tubule diameter is a dynamic property narrowing with age or sclerosing in response to caries. Nonetheless, as Fogel *et al*. (1988) and Garberoglio and Brännström (1976) have shown, tubule diameter and density is always going to be greatest in inner dentine, thus diffusion will be much faster in this region while slowing as it moves outward. Thus, the further bacteria penetrate along a tubule towards the periodontal ligament the better their chance of surviving the challenge of the calcium hydroxide medication.

Smear layer is another obstacle to the diffusion of calcium hydroxide. Smear layer is a thin layer of organic and inorganic debris that forms on root canal walls as a result of root canal instrumentation procedures. Smear layer often occludes and extends into dentinal tubules. Ethylenediaminetetraacetic acid (EDTA) irrigation is commonly recommended to remove this smear layer in order to open up dentinal tubules and allow better diffusion of endodontic medicaments

through dentinal tubules. Interestingly, Galvan *et al.* (1994) and Tao *et al.* (1991) found when smear layer was removed, dentine became initially less permeable to calcium hydroxide. This was thought to be due to precipitations within the tubules. However, other studies have shown the opposite with an improvement in permeability following smear layer removal (Abbott *et al*., 1989, Ørstavik and Haapasalo, 1990).

High levels of diffusion is an essential aspect of a medicament. If this does not occur, or occurs too slowly, bacteria deep within the recesses of the canal system may not be exposed to sufficient concentration of medicament to affect killing or the bacteria may have time to adapt to a gradual change in environmental conditions. This is of concern because Flahaut *et al.* (1997) have shown that pre-exposure to a sub-lethal increase in pH may induce *E. faecalis* to adapt and become resistant to a normally lethal pH. This situation might be experienced *in vivo* as the hydroxyl ions of calcium hydroxide diffuse throughout the pulp-dentine complex resulting in only a slow rise in pH in some locations (Nerwich *et al*., 1993)

1.5.2.3 Buffering

Chemical interactions can adversely affect the antibacterial efficacy of medicaments. A buffering effect of dentine has been demonstrated which inhibits a change in pH when challenged by an acid or base (Haapasalo *et al*., 2007).

Haapasalo *et al*. (2000) crushed dentine and sterilized the resulting powder then mixed and incubated it with *E. faecalis* before adding a normally lethal dose of calcium hydroxide solution. Surprisingly, calcium hydroxide had its antibacterial effect completely abolished in the presence of dentine powder. Other disinfectants, 0.5% chlorhexidine and 1% sodium hypochlorite, had their antibacterial effect reduced but not eliminated. This result was attributed to the buffering effect of the inorganic component of dentine, namely the presence of carbonate ions. While not an exact simulation of the situation within a root canal because of the increased surface area of powder and the use of a saturated

solution as opposed to a paste with a reservoir of undissolved solute, this study suggests an inhibitory effect of dentine on calcium hydroxide efficacy.

Portenier *et al.* (2001) investigated this phenomenon further by investigating the effect of the inorganic component of dentine compared with whole dentine. Some medicaments such as chlorhexidine and iodine were not affected by hydroxyapatite but were reduced in effectiveness by whole dentine implicating the organic component of dentine. This organic component (collagen) makes up some 20% by mass of whole dentine. However, the organic component of dentine is potentially not the only source of organic material in the root canal. For example, other sources may include necrotic tissue, microbial biomass and inflammatory exudate. Portenier *et al.* (2001) further investigated a possible effect of organic material. Using bovine serum albumin (BSA) as surrogate for organic material, they found that BSA also completely inactivated any effect of calcium hydroxide on *E. faecalis*.

It must be emphasized that these *in vitro* studies poorly represent the *in vivo* situation, but they appear to show an interaction between calcium hydroxide and likely components of the root canal system. Such interaction combined with the poor solubility of calcium hydroxide and its reliance on diffusion, all helps explain the observations of Nerwich *et al.* (1993), that the pH in outer dentine only rises slowly and does not achieve the maximum pH attributed to calcium hydroxide. Such a slow rise may provide bacteria in the outer reaches of roots time to adapt and activate stress response mechanisms to deal with increases in pH imparted by calcium hydroxide.

1.5.2.4 Time

The recommended time to leave medicaments in place can vary and is mostly dependant on the physical properties of the medicament. The recommendations tend to be empirical and often disregard tooth factors that may have an influence on a medicaments diffusion and action such as age of the tooth, status of the tooth prior to treatment and the quality of debridement and temporary restoration (Abbott, 1990). Furthermore, different studies measuring different

parameters have generated different recommendations. For example, while Nerwich *et al.* (1993) has shown peak pH levels may take 2-3 weeks to be reached, Sjögren *et al.* (1991) showed a one week dressing of calcium hydroxide to be effective when microbiological sampling and culturing was used.

Some medicaments rapidly diffuse while others are slower. The longevity and diffusion of calcium hydroxide in the root canal system can change depending on its consistency and whether other additives are used. Calcium hydroxide has been mixed with a range of vehicles including water, methylcellulose, glycerine and other oily solutes (Fava and Saunders, 1999). Water–based vehicles allow faster diffusion of calcium hydroxide into tubules especially if it is a thin mix of calcium hydroxide. In contrast thicker mixes of more viscous vehicles retain the medicament in the canal for longer but diffuse slower (Behnen *et al*., 2001, Safavi and Nakayama, 2000). However, altering the diffusion of a medicament can negatively impact its principal action which is to eliminate bacteria especially deep within dentinal tubules or accessory canals. The desired effect of a medicament will influence how long it should be left in a canal. For example, within three weeks, calcium hydroxide may kill a certain number of bacteria. But if the aim is apexogenesis it will need to be left in place for much longer. Thus, it is important that practitioners take into account the medicament (type and vehicle), tooth factors (infected status, age, resorption, degree of debridement) and desired effect when making clinical decisions.

Clearly, calcium hydroxide placed as an inter-appointment dressing during root canal treatment faces a number of challenges to overcome and exert its desired antimicrobial effect. Given that this effect is pH dependant, the medicament must diffuse in sufficient concentration and in sufficient time in order to exert its action. The obstacles discussed may result in a failure to reach sufficient pH and/or produce a slow rate of increase in pH to some outer areas of the pulp-dentine complex. In combination with microbial resistance mechanisms, that will be discussed next, these limitations of calcium hydroxide dressing may result in an incomplete disinfection of the root canal system and a persistence of highly resistant bacteria.

1.6 Survival Mechanisms of *E. faecalis* Biofilm Against Calcium Hydroxide

The bacterial susceptibility to calcium hydroxide is essentially a susceptibility to an increase in pH caused by hydroxyl ions. Various virulence factors may allow some bacteria to better survive a high pH environment. Some of these factors form part of a general bacterial response to stress while other factors may be specific to certain species. The exact mechanisms by which bacteria survive a pH challenge are not well understood but research is ongoing and is starting to elucidate some of the mechanisms.

1.6.1 Innate Resistance to High pH

E. faecalis appears to have an innate resistance to calcium hydroxide. It has been shown to be more tolerant to high pH challenge compared to other bacteria found in endodontic infections. Byström *et al.* (1985) demonstrated this when an *E. faecalis* isolate survived longer than other endodontic bacterial isolates in a calcium hydroxide solution up to pH 11.5 .

Flahaut *et al* (1997) have demonstrated an adaptive response of *E. faecalis* when exposed to an alkaline stress. When exposed to pH 10.5 for 30 min, 83.3% of adapted cells survived. When these surviving cells were exposed to a further increase in pH up to 11.9 nearly 100% survived over 30 min. In contrast, when grown and only exposed to an optimal growth pH of 7.2 prior to exposure to the high pH of 11.9 over 99.9% of bacteria died within 30 min. From this result it was concluded the exposure to pH 10.5 initiated some sort of adaptive stress response mechanism that improved survival at pH 11.9.

Evans *et al.* (2002) mimicked this experiment by exposing a planktonic culture of *E. faecalis* to a calcium hydroxide solution at pH 10.3 for 30 min and then increased the pH to 11.5. It was found pretreatment conferred no increase in resistance to pH.

The methodology of these two studies is significant to any potential clinical relevance. Depending on the position of the bacteria in some areas of the pulp-

dentine complex the rise in pH may be slow providing time for the bacteria to adapt similar to Flahaut *et al*. (1997). However, clinically, a calcium hydroxide medicament is normally placed for longer than one hour and prolonged exposure to high pH may result in greater rates of killing than was demonstrated by Flahaut *et al.* (1997). The fundamental difference between the work of Evans *et al.* (2002) and Flahaut *et al.* (1997) was the respective use of a saturated solution of calcium hydroxide versus a solution of Brain Heart Infusion broth (BHI) adjusted to a high pH with sodium hydroxide. In the work of Flahaut *et al.* (1997) bacteria may still have been able to derive energy from the BHI with which to manage the alkaline insult. Clinically, in the root canal, bacterial enzymes such as gelatinase may still have provided *E. faecalis* with energy for which to cope and adapt to an alkaline challenge.

Evans *et al.* (2002) also suggested a potential mechanism for survival of *E. faecalis* at higher pH. When a proton pump inhibitor was added to the calcium hydroxide solution, greater killing efficacy was obtained than without the inhibitor at pH 11.1. This seems to suggest that proton pumps located in the cell membrane of *E. faecalis* may be involved in regulating the internal pH and thus survival when challenged by an alkaline environment. Cytoplasmic pH regulation is essential for normal cellular activity, function and survival (Kakinuma, 1998). In alkaline environments, cytoplasmic pH is generally acidified by Na^+/H^+ and K^+/H^+ antiporter proton pumps that move H^+ into cells and the respective cations out utilizing osmotic differences in solute fluxes (Booth, 1985, Kakinuma, 1998). Appelbe and Sedgley (2007) have shown an increased expression of a proton pump gene, *napA* which codes for a Na^+/H^+ antiporter, when *E. faecalis* was exposed to pH 10. Kakinuma and Igarashi (1995) have described a somewhat different Kep system in *Enterococcus hirae* whereby an active energy-linked K^+/H^+ antiporter acidifies the cytoplasm when exposed to high pH rather than relying on osmotic differences to drive the proton pumps.

1.6.2 Starvation Resistance

E. faecalis has been demonstrated, both *in-vitro* and *ex vivo,* to be capable of surviving several months without significant nutrition (Figdor *et al*., 2003, Sedgley *et al*., 2005d). Sedgley *et al.* (2005d) demonstrated *E. faecalis* survival in extracted root filled teeth after 12 months. Such survival is significant because nutrient availability within root filled teeth, in which *E. faecalis* is commonly found, is likely to be extremely limited. It may vary from canal to canal based on the quality of the obturation and apical leakage, but with reasonable certainty it can be assumed that the available nutrition is less than in an unfilled canal. *E. faecalis* has been shown by Portenier *et al.* (2005) to survive in greater numbers in a starved state when exposed to saturated calcium hydroxide compared to the same bacteria during an exponential phase of growth with plentiful nutrient. The bacteria in this study were grown planktonically and it was concluded that the same stress responses that were allowing survival of the bacteria under starvation conditions may also be associated with resistance to alkaline challenge.

1.6.3 Tubule invasion

The invasion of dentinal tubules by *E. faecalis* is well established (Haapasalo and Ørstavik, 1987, Love, 2001). Love (2001) demonstrated that *E. faecalis* also invaded tubules in the presence of serum. This is significant because invasion of tubules by *Streptococcus gordonii* and *Streptococcus mutans* were inhibited by the presence of serum. The fact that *E. faecalis* was capable not only provides evidence of invasion in a more realistic *in vitro* experiment but the presence of serum could be a means of continued nutrition and thus survival within the tubules.

1.6.4 Biofilm

Distel *et al.* (2002) has shown in an *in vitro* study, that *E. faecalis* can form biofilm structures and re-contaminate a sterile root canal in the presence of a calcium hydroxide dressing. After 77 days, all test samples showed *E. faecalis* encompassed in a heavy extra-cellular matrix that was adherent to dentine walls. They suggested this represented a well-developed biofilm. Interestingly,

however, they were unable to show a difference in protein expression between the bacteria taken from the biofilm and those from the inoculating planktonic solution. Nonetheless this provides some evidence that calcium hydroxide may stimulate the formation of biofilms which may form part of the organisms stress response to high pH. A similar observation was reported by Kayaoglu *et al.* (2005) when an increased adhesion to collagen was seen upon increasing the pH to 8.5. This may represent the early stages of biofilm development. Aggregation substance and *ace* genes were suggested as possible initiators.

Bacteria within biofilms have been shown to be up to 1000 times more resistant to some antimicrobials than the same bacteria in a planktonic phase (Svensåter and Bergenholtz, 2004). Therefore the ability to form a biofilm structure could be considered a virulence factor as it aids in bacterial survival. Brandle *et al.* (2008) showed that such attachment to dentine enhanced the survival of *E. faecalis* significantly when exposed to short durations of calcium hydroxide. Chávez de Paz *et al.* (2007) has shown that *E. faecalis* biofilm was much more resistant to a pH 10.5 solution than its planktonic counterpart when exposed for 4 hours. In contrast to this finding, Abdullah *et al.* (2005) demonstrated that with a higher pH of 12.3 over 60 minutes, no difference in survival occurred between biofilm and planktonic states. However, there was only a two thirds reduction in colony forming units (CFU) over the exposure period, so exposure of a more clinically relevant duration, such as one week or more, may have shown a different result.

1.6.5 Viable But Non-Culturable (VBNC)

Although the viable but non-culturable (VBNC) state remains controversial, it has been suggested as a survival mechanism in response to environmental stresses. In this state, cellular modifications provide protections against challenges like low nutrient levels, low and high temperatures and extreme pH (Lleo *et al*., 2000). In the VBNC state it has been suggested bacteria lose the ability to divide and grow on culture media. However, such a characteristic makes it very difficult to discern dead bacteria from those that have maintained

their viability but are not replicating. Studies using Reverse Transcriptase PCR have provided some evidence of short-lived mRNA amongst possible populations of VBNC bacteria (Lleo *et al*., 2000, Williams *et al*., 2006). Detection of such mRNA is suggestive of viable bacteria. However, definitive proof of bacteria within this state having the ability to recover and grow again has not been demonstrated (Portenier *et al*., 2003).

1.7 Justification for this Study

E. faecalis is a bacteria commonly associated with failed endodontically treated teeth (Molander *et al*., 1998, Sundqvist *et al*., 1998). Retreatment of these teeth has a lower success rate than treatment of primary endodontic disease (Sjögren *et al*., 1990). A degree of inherent resistance and adaptive stress response mechanisms may possibly combine to create an *E. faecalis* phenotype resistant to endodontic treatment while other bacteria are eliminated. Calcium hydroxide is a commonly used endodontic medicament that exerts its action through a localized increase in pH within the root canal. However, calcium hydroxide has a number of limitations. In particular, its use relies on its diffusion through the pulp-dentine complex to cause a rise in pH (Haapasalo *et al*., 2000). Furthermore, this rise in pH may potentially be buffered by dentine itself. These two factors combined may result in some areas within the root canal system, such as peripheral dentinal tubules or accessory canals, only reaching a high pH slowly (Nerwich *et al*., 1993). A slow rise in pH, as this study aims to simulate, may allow time for an adaptive response by *E. faecalis* bacteria located within these outer reaches. Such an adaptive response may result in a more resistant phenotype. Development of a resistant phenotype following calcium hydroxide medication may be harmful as it may enhance the chances of *E. faecalis* surviving ongoing and possibly future endodontic therapy with a concomitant decrease in the chances for a successful outcome (Sjögren *et al*., 1997, Sundqvist *et al*., 1998).

1.8 Aim

The aim of this study was to compare the survival of an *Enterococcus faecalis* biofilm grown on dentine when exposed to rapid or slow increases in external pH.

2. Methods and Materials

2.1 Test Organism

Enterococcus faecalis (V583, ATCC 700802) was purchased from ATCC (Cryosite, Australia) and stored as frozen stock cultures in 40% v/v glycerol at -80^{0}C. Pure cultures were grown on Todd Hewitt Broth Agar (Oxoid, Victoria, Australia) and were regularly sub-cultured to maintain purity. Purity was checked by periodic Gram staining.

For the inoculation of the flow cells, overnight broths of *E. faecalis* were made using 50 ml of Todd Hewitt Broth (THB- Oxoid, Victoria, Australia). Gram staining of the overnight broth was always carried out prior to use to confirm purity.

2.2 Dentine Disc Preparation

Dentine discs were created from extracted single rooted teeth and large palatal roots of molars. Ethical approval was granted by the University of Adelaide Research Ethics Committee (Project No: H-121-2009). The age and disease status of the teeth was unknown. Extracted teeth were stored in 0.9% saline. The crowns of the teeth and the apical thirds of the roots were sectioned and discarded. The remaining root specimens were sectioned horizontally in 1mm thick sections with a slow-speed diamond saw.

These disc specimens then had remnant organic pulp and periodontal ligament tissue mechanically removed with high speed, 169 tungsten carbide burs (Komet, Besigheim, Germany) and Sof-Lex discs (3M, Minnesota, USA). This was carried out under 2.5 x magnification with illumination. Specimens were then placed in 15% EDTA solution for 1 min to reduce debris and potential smear layer.

Discs were subsequently rinsed with 0.9% saline (w/v) and then stored dry until use.

2.3 Dentine Disc Serial Transfer Biofilm Growth Model

Two different biofilm growth models were investigated. The first model involved serial transfer of inoculated dentine discs to fresh THB solution every 48 hrs to stimulate constant biofilm growth on the dentine disc surface over a four-week period.

THB was prepared at a concentration of 50% (18.2 grams/litre) compared to that of the manufacturer's instructions. Pilot experiments determined this concentration supported growth of *E. faecalis* close to an optical density (OD) (560 nm)=1. This was considered a sufficient bacterial load to support the establishment of a biofilm.

5ml of THB and wire hooks, made from 0.016" stainless steel wire, were placed in 5 ml polypropylene tubes (TechnoPlas, South Australia , Australia). The tubes were sterilized by autoclaving at 121^0C for 15 min. Sterility was confirmed by the absence of turbidity following incubation at 37^0C for 48 hrs .

Discs were sterilized with ethylene oxide at the at the Flinders Medical Centre, Central Sterilising facility (Adelaide, Australia). They were then placed aseptically onto the wire hooks.

An overnight broth of *E. faecalis* (V583) was adjusted to OD=1 (560 nm) with sterile 0.9% saline (w/v). Pure culture was confirmed with Gram staining. 500 μl was inoculated into each tube containing disc specimens to produce 1:10 dilution. The tubes were then incubated at 37^0C for 48 hrs.

Every 48 hrs the wire hooks were used to serially transfer discs to new sterile tubes containing fresh THB. No further inoculation of the broth occurred because bacteria attached to the wire and discs provided a sufficient inoculum to produce turbidity of the broth within 48 hrs. Fresh broth was provided to ensure sufficient nutrient for continued growth and development of the biofilm.

The serial transfer occurred for up to 4 weeks. At 1, 2, 3 and 4 weeks discs were removed from the experiment and were fixed for SEM analysis of the biofilms.

2.4 pH Adjusted Buffer Soultions Used for Alkaline Test Agents

The buffer used in the experiments was a glycine (Sigma, Seoul, Korea)/NaCl and NaOH buffer solution which has a stable pH buffering range between 8.5 and 12.8 (Lynch *et al*, 1969). This buffer was chosen because its range covered all the pHs needed for this study. Buffer was used so the pH of the test agents was not reduced by the nutrient metabolism of the experimental biofilms.

Buffer components were mixed accordingly to match the respective pHs needed for each experiment (Lynch *et al*, 1969). THB powder was then added to this buffer according to the manufacturer's recommended concentration (36.4 g/L) to make a THB buffered solution. The solution was then sterilized in an autoclave. The pH was monitored and accepted if it was in the range of the target pH +/- 0.2. It was found that THB reduced the pH of the buffered solutions. The desired pH was therefore achieved by addition of a strong alkali, 3 N KOH. The same pH meter was always used and was always calibrated prior to use.

pH testing carried out over 3 days showed that sterile buffered THB had a stable pH.

2.5 Planktonic Growth Curves

Growth curves were made to determine the growth rate of planktonic *E. faecalis* when exposed to alkaline pH and confirm that buffered THB supported growth.

Buffered THB was made to pH 8.5, 9.5, 10.5 and 11.5. A control (unbuffered) solution of unbuffered THB was made using only demineralised water. Solutions were sterilized in an autoclave and following this had their pH tested and adjusted as described above.

An overnight broth of *E. faecalis* in THB gave an OD (560 nm) of 1.17.

20 ml of buffered THB at each pH was added to sterile tubes. 2 ml of overnight broth was inoculated into each tube. 1ml was removed for OD analysis (560 nm) every hour for the first 6 hrs and also at 24 and 48 hrs.

The pH of the growth medium was also checked after the experiments to monitor the effect of the addition of the inoculum on pH.

2.6 Flow Cell Biofilm Growth Model

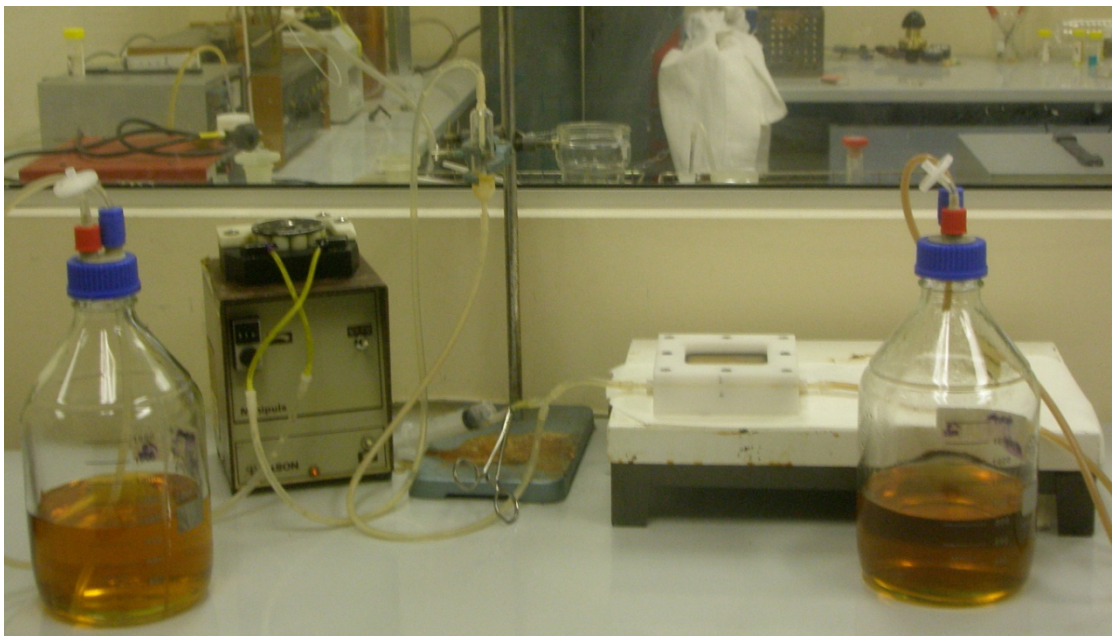


Figure 1: Flow cell system

A flow cell biofilm model that had been previously designed, tested and verified was ultimately used to establish biofilms for pH experimentation because it produced a more confluent and dense biofilm than the serial transfer model described in Section 2.3 over a four-week growth phase (Plutzer, 2009). The system consisted of a nutrient reservoir, single channel peristaltic pump (Gilson, Wisconsin, USA) flow cell and waste vessel connected by silicone tubing (Fig 1). A maximum of 13 dentine discs were placed within the flow cell using modified endodontic files inserted into recesses in the base of the flow cell (Fig 2).

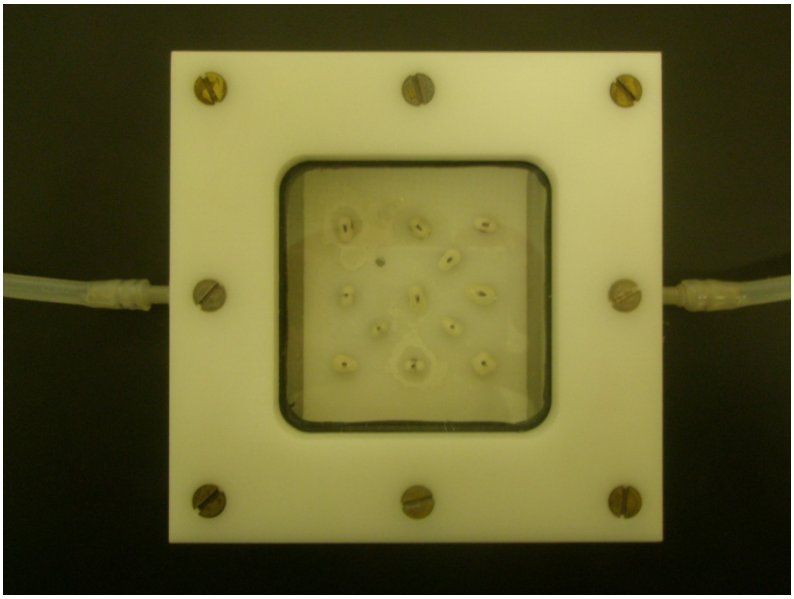


Figure 2: Flow cell with dentine discs in place

The flow cell and discs were sterilized with ethylene oxide at the Flinders Medical Centre, Central Sterilising facility (Adelaide, Australia). The nutrient reservoir, tubing and waste vessel were sterilized by autoclaving at 121^0C for 15 min and were attached to the flow cell in an aseptic manner. The flow cell was placed on a slide drying bed (Ratek, Victoria Australia) to maintain a constant temperature of the flow cell at 37^0C.

Todd Hewitt Broth was pumped into the flow cell and was then left for 24 hours to check sterility. Sterility was taken as the absence of broth turbidity or the absence of bacteria following plating of broth collected in the waste vessel.

The flow cell system was inoculated with 50 ml of an overnight broth of *E. faecalis* (ATCC V587) via a syringe injection port located approximately 3-4 cm upstream of the flow cell. After inoculation the pump was turned off for approximately 8 hrs to allow for bacterial growth. When the pump was turned back on, a flow rate of 30 ml/hour was established. Given the flow cell volume was 50 ml, this equated to a dilution rate of 0.6 hr^{-1}.

The pump and system was continuously run for four weeks. 2L nutrient bottles and waste collection bottles were replaced approximately every 2.5 days. Waste broth was regularly checked for contamination by Gram staining.

2.7 Alkaline pH Exposure

After four weeks of biofilm growth, the growth phase ended and the experimental phase began. The experimental phase ran for one week. The first flow cell acted as a control, whereby the same THB used in the growth phase continued to be pumped through the flow cell for a further week.

Each subsequent flow cell was alternately assigned one of two experimental groups (Table 1):

Rapid Rate of pH increase

- During the first three days of the experimental phase unbuffered THB was continually pumped through the flow cell. On day four, buffered THB of the maximum pH (either 11.5 or 12.5) was rapidly introduced into the flow cell and maintained at this pH for 4 days (Tables 2 & 3).

Slow Rate of pH Increase

- During the first three days of the experimental phase, the pH was increased daily with buffered THB until the maximum pH (either 11.5 or 12.5) was reached on day 4. This maximum pH was maintained for a further 4 days (Tables 2 & 3).

For flow cells being exposed to the rapid increase in pH, the flow cells were initially pumped dry before immediately filling with the buffered THB. This process took approx 5 min and was designed to prevent the initial dilution of the pH of the buffered THB. Buffered THB was, for the remainder of the experimental phase, continuously pumped through the flow cells at the same rate as during the growth phase.

	Number of Flow Cells
Control	1
Slow Increase to pH 11.5	1
Rapid Increase to pH 11.5	1
Slow Increase to pH 12.5	2
Rapid Increase to pH 12.5	2

Table 1: Flow cell allocation

	Day 1	2	3	4	5	6	7
Control	THB*	THB	THB	THB	THB	THB	THB
Rapid Increase	THB	THB	THB	pH 11.5	pH 11.5	pH 11.5	pH 11.5
Slow Increase	pH 8.5	pH 9.5	pH 10.5	pH 11.5	pH 11.5	pH 11.5	pH 11.5

THB refers to unbuffered THB prepared with demineralised water (pH 8.1). All other pHs refer to THB buffered to the pH indicated.

Table 2: Experimental protocol showing pH Increase (pH 11.5=max)

	Day 1	2	3	4	5	6	7
Control	THB*	THB	THB	THB	THB	THB	THB
Rapid Increase	THB	THB	THB	pH 12.5	pH 12.5	pH 12.5	pH 12.5
Slow Increase	pH 9.5	pH 10.5	pH 11.5	pH 12.5	pH 12.5	pH 12.5	pH 12.5

THB refers to unbuffered THB prepared with demineralised water. All other pHs refer to THB buffered to the pH indicated.

Table 3: Experimental protocol showing pH Increase (pH 12.5=max)

The pH of the buffered THB was checked immediately before its use to confirm the pH. Also, the pH of fresh waste from the flow cell was periodically monitored to check that the target pH was being reached inside the flow cell.

2.8 Biofilm Examination and Measurement

At the end of the seven day experimental phase the flow cell was disassembled. Discs were aseptically removed from the flow cell and placed in tubes containing 2 ml of sterile 0.9% saline for rinsing. Care was taken not to disrupt the surface biofilm. Discs were agitated on a microplate shaker (Titertek, Pforzheim, Germany) for 15 sec to remove unattached bacteria. This process was repeated twice.

Discs were then allocated randomly to one of three methods of examination and processing (Table 4):

1. Viability plating
2. Scanning Electron Microscopy (SEM)
3. Confocal Laser Microscopy (CLSM)

	Number of Discs
Viability Culture	7-8
SEM	2
CLSM	2

Table 4: Disc allocation per flow cell

2.8.1 Viability Plating

Discs were placed in 2 ml of fresh sterile 0.9% saline. Biofilm was detached from the discs into the saline by sonication with a Soniprobe in 3 x 5 second bursts (Dawe Instruments Ltd, England). As well as applying sonication in short bursts, a low 1 ampere power setting was used in order to remove bacteria from the disc while avoiding cell lysis. For the experiment comparing a rapid versus slow rise to maximum pH of 11.5 and the first experiment comparing a maximum pH of 12.5, one disc was added to 2ml of saline for biofilm harvesting and viability plating. When the pH 12.5 experiment was repeated 3 discs were added to 2ml of saline. This reduced the number of samples per flow cell but the aim of this was to increase the bacterial numbers per sample.

Serial dilution of the biofilm suspension was followed by plating 100µl aliquots in duplicate onto THB agar plates. Plates were incubated aerobically at 37^0C for 24 hrs before colonies were counted. The viability counts were then converted to colony forming units (CFU) /ml of biofilm suspension.

Cellular protein levels were measured to normalize the viability counts to the total amount of bacterial biofilm harvested per disc. 100µl of 0.1 M NaOH was added to a 0.9ml sample of each biofilm suspension and boiled for 30 min. 150µl of each sample was then pipetted into a microplate well and mixed with 150µl of Coomassie Plus™ Protein Assay Reagent (Thermo Scientific, Illinois, USA). The microtitre plate was then shaken and read at 595nm by a microplate reader (Bio-tek, Vermont, USA). Protein concentrations were standardized against

known concentration of bovine serum albumin (BSA) standards which were assayed at the same time as biofilm suspensions.

CFU per mg of protein were then determined to allow comparisons of viability between control, slow and rapid groups.

2.8.4 Scanning Electron Microscopy

Discs assigned for SEM were stored in electron microscopy fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS + 4% sucrose). Sonication was not carried out on these discs.

For processing discs were first washed with PBS + 4% sucrose and then dehydrated with increasing concentrations of ethanol

1. 70% ethanol- 15 min
2. 90% ethanol- 15 min
3. 95% ethanol- 15 min
4. 100% ethanol- 3x 10 min changes

Dehydration was followed by critical point drying and then coating with platinum. Disc specimens were analyzed using a Philips XL30 scanning electron microscope in the range of 10,000-15,000 x magnification. Discs were assessed for biofilm presence, confluence, density and arrangement.

2.8.3 Confocal Laser Microscopy

Following rinsing, discs assigned for confocal microscopy were placed in 1 ml of sterile saline 0.9%. Again, sonication was not carried out on these discs. The biofilm on the disc was stained with the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, California, USA). This kit is designed to stain live bacteria green with a SYTO®9 stain and dead bacteria red with propidium iodide stain.

The staining method used was a minor modification of the manufacturer's instructions due to the fact biofilm attached to dentine and not a planktonic suspension was being tested:

1. 1.5µl of SYTO® 9 and propidium iodide stains were mixed thoroughly in a microfuge tube.
2. The 3µl was added to the 1ml of saline containing the disc specimen and the tube was gently agitated to mix stain and saline.
3. The tube was incubated in the dark for 15 min
4. The stain was removed from the tube using a pipette and the disc rinsed 3 times with fresh saline

After staining, the disc was placed in specially designed apparatus. This consisted of a microscope slide with a rubber "O" ring glued on using polysiloxane impression material. The disc sample was place inside the O-ring on the slide. The rubber "O" ring was filled to its surface with saline and a slide cover was placed on top. This was retained by wax (Fig 3).

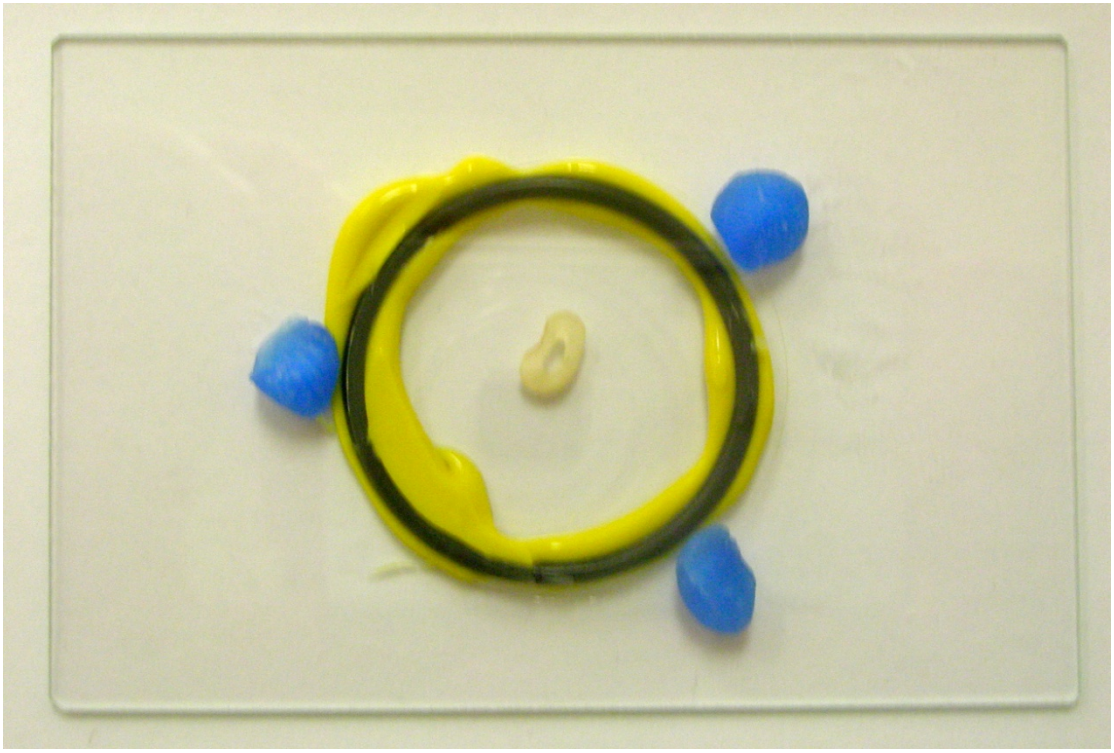


Figure 3: CLSM slide

The confocal specimen was then immediately taken for analysis with a Leica SP5 confocal laser microscope (Leica Microsystems, Mannheim, Germany). A 488 nm

argon laser was used to excite the fluorescent stain with emission filters set between 510 - 540 nm for green fluorescence and 620 - 650 nm for red fluorescence. Biofilm presence and confluence was observed. The relative presence of green and red staining was noted.

2.9 Statistical Analysis

Statistical analysis was carried out by applying the nonparametric Wilcoxon test to the results of bacterial viability counts (CFU/mg protein) (Section 2.8.1). Bacterial viability after rapid and slow increases to maximum pHs of either 11.5 or 12.5 were compared. Viability of bacteria harvested from flow cells exposed to the maximum pH of 12.5 was also directly compared to those exposed to the maximum pH of 11.5. The pH 12.5 experiment was replicated twice. However, due to the change in sampling method whereby three discs per sample were harvested (Section 2.8.1) in the second experiment, this experiment could not be directly compared with the first pH 12.5 experiment.

3. Results

3.1 Dentine Discs

SEM images taken of discs after initial preparation but prior to biofilm growth showed a flat surface with cracks and fissures present (Fig 4). A firmly adherent dentine smear layer covered the surface of the discs probably as a result of mechanical disc cutting procedures. This was considered an acceptable surface for this study. Further treatment of the discs to remove smear layer using agents possessing antimicrobial properties, such as EDTA or sodium hypochlorite, was considered undesirable because preliminary studies had shown a possible residual antimicrobial effect.

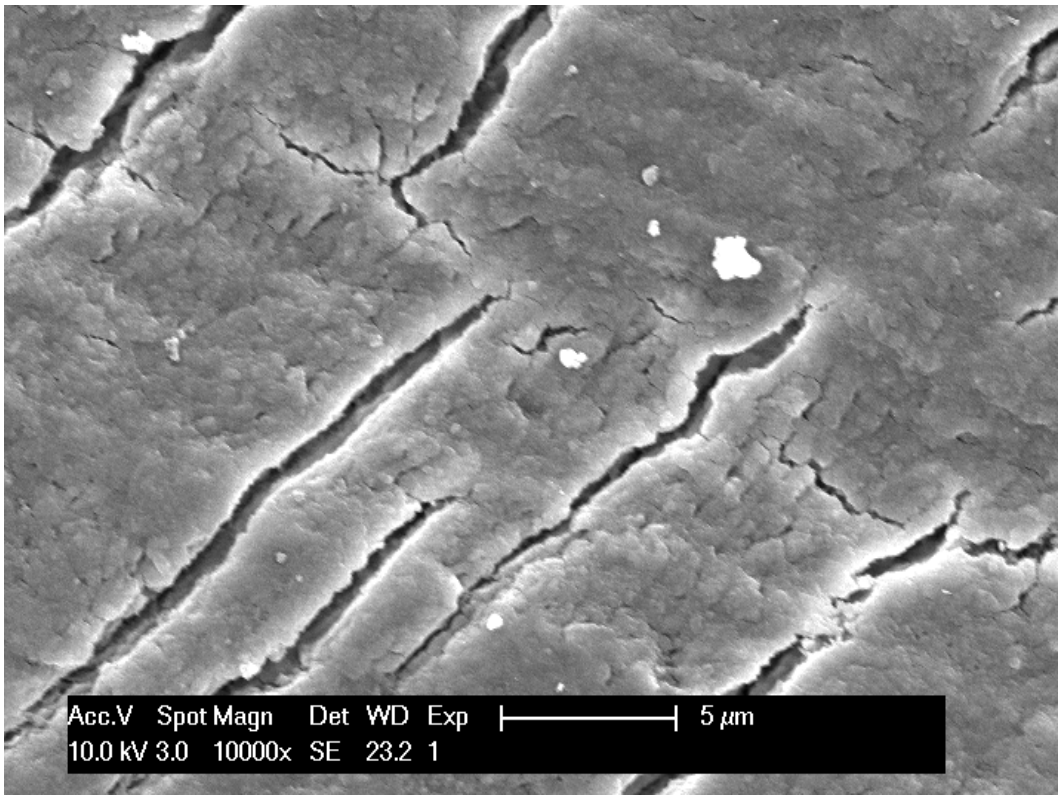
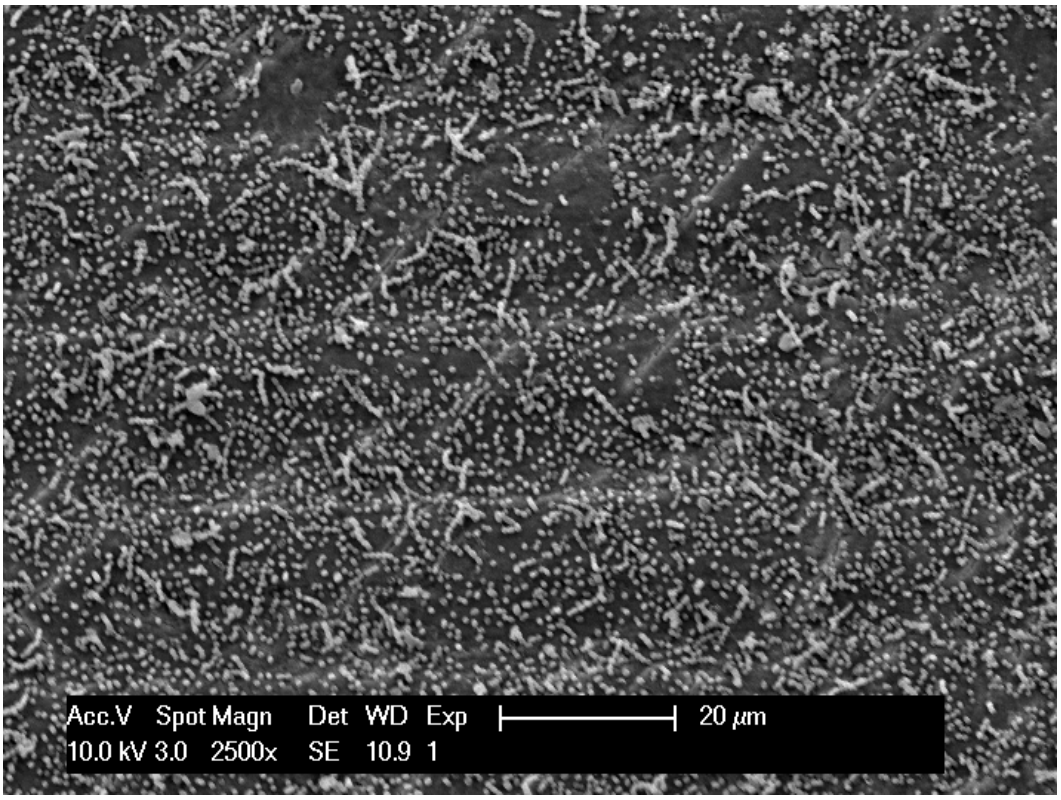


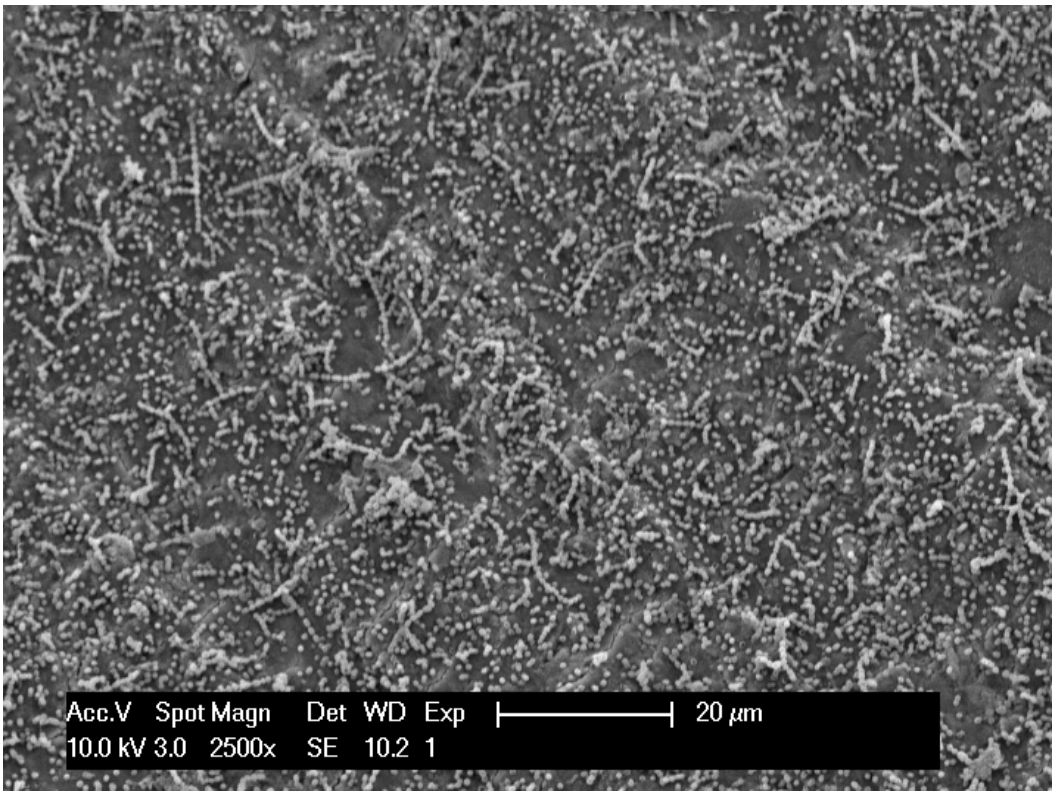
Figure 4: SEM image of the horizontal surface of the dentine disc prior to biofilm growth (10,000 x magnification)

3.2 Dentine Disc Serial Transfer Biofilm Growth Model

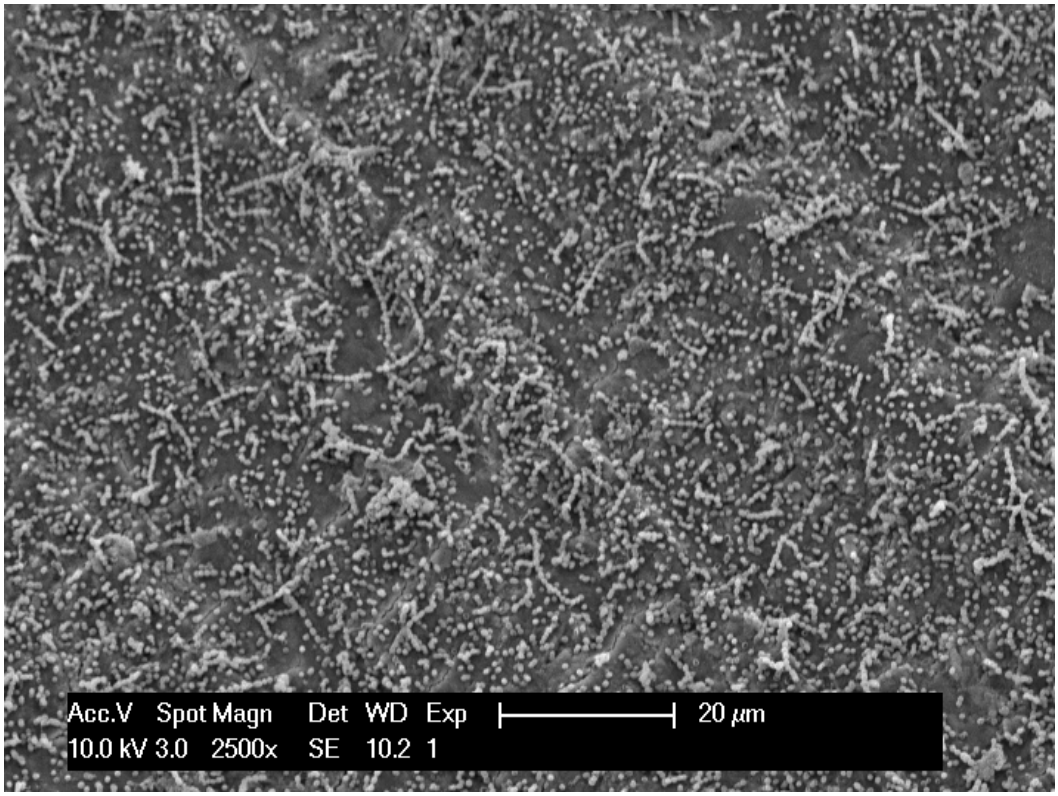
a.



b.



c.



d.

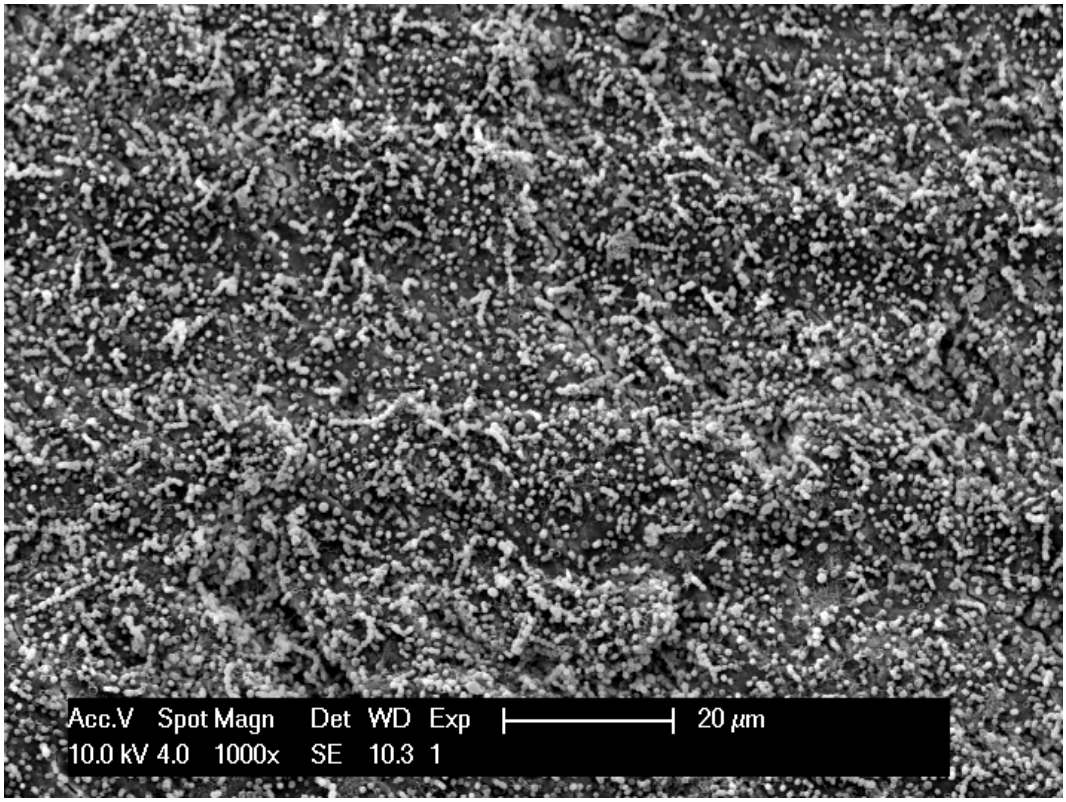


Figure 5: SEM images of biofilm development over 4 weeks using a dentine disc serial transfer biofilm model. Images of comparable scale show biofilm development over (a) 1 week, (b) 2 weeks, (c) 3 week, (d) 4 week

SEM was used to evaluate the biofilm at one week intervals over four weeks (Fig 5). After one week attachment to dentine discs, individual cells and groups arranged as short chains were evident. The number of attached cells and chains increased by week two and extra-cellular rope-like structures became evident inter-cellularly and between cells and the dentine surface. At three weeks, the development of larger clumps of bacteria within an amorphous extracellular substance became apparent although such clumps were sparse. SEM images at week four revealed the most confluent biofilm. Aggregates of bacteria were more evident and extensive attachment between neighbouring cells and between cells and dentine was apparent via extra-cellular material. However, while most of the dentine surface was covered in cells, it was not a dense, multilayered biofilm.

While this biofilm growth model did initiate the development of a biofilm it was slow. With further time a denser biofilm may have developed. However, for practical reasons this biofilm model was abandoned and a proven flow cell model was used that had previously been shown to produce confluent, dense, multilayered biofilms over four weeks.

3.3 Planktonic Growth Curves

Media	THB pH at T_0	THB pH at $T_{72\ hrs}$
Control THB	8.11	8
pH 8.5	8.66	8.66
pH 9.5	9.56	9.61
pH 10.5	10.47	10.42
pH 11.5	11.7	11.3

Table 5: Stability of the pH of buffered THB media over a 72 hr period. Control THB is unbuffered media prepared with demineralised water.

pH readings of the buffered THB solutions were shown to be acceptably stable over the course of three days with only minor changes (Table 5). The pH 11.5 solution showed the most change in its pH during this test, ΔpH = -0.4. However, subsequent use showed this result to be an aberration and the pH 11.5 buffer was as stable as other buffered solutions.

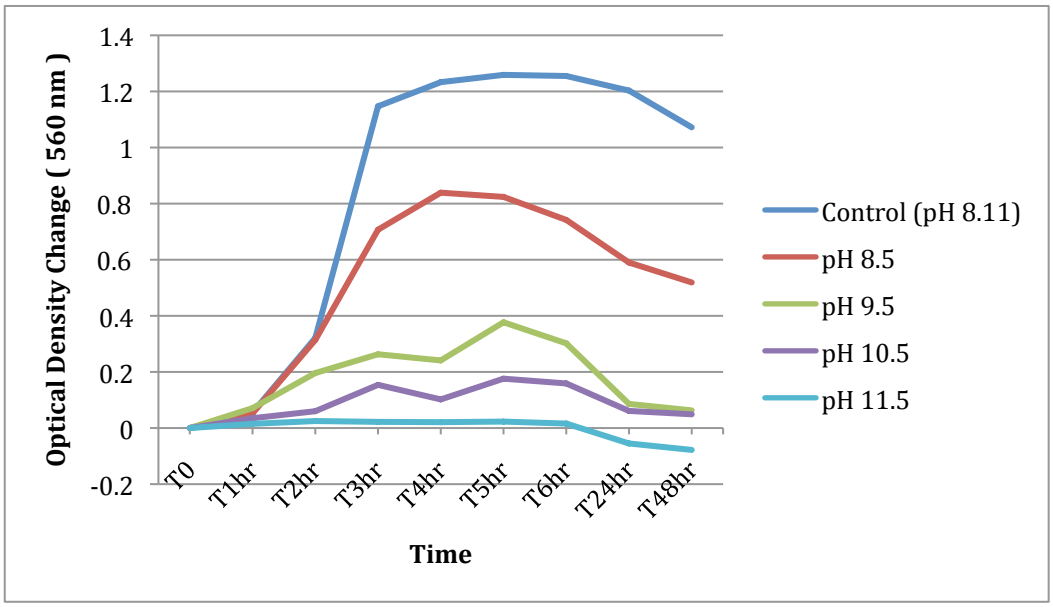


Figure 6: Planktonic growth of *E. faecalis* at different pH in buffered THB (OD=560nm)

Figure 6 shows that the buffered THB supported a typical bacterial growth pattern as measured by optical density (560nm). Comparison between the control and buffer pH 8.5 shows this most clearly with both solutions demonstrating initial log phase growth of bacteria followed by a plateau (stationary phase), followed by a slow reduction in optical density.

Media	Pre-incoulation pH	Post-inoculation pH (48hrs)
Control	8.11	7.22
pH 8.5	8.66	8.23
pH 9.5	9.56	9.26
pH 10.5	10.47	10.08
pH 11.5	11.7	10.72

Table 6: Change in pH of buffered and control THB media following bacterial inoculation after 48 hrs

Table 6 shows the reduction in pH that occurred during the planktonic growth curve experiment following inoculation by *E. faecalis*. This is likely to be due to the production of acidic end-products as a result of nutrient metabolism. The reduction in pH of the buffered THB solutions pH 8.5 - 10.5 ranged from -0.3 to -0.43. The pH 11.5 solution appears to have reduced in pH the most by -0.98. However, the buffered THB used was the same as the media tested in Table 5. If the passive decrease in pH 11.5 noted in Table 5, without inoculation of bacteria is adjusted for, the overall reduction as a result of bacterial buffering in Table 2 is -0.58. This result is more in line with the change in pH of the buffered media prepared to pH 8.5 - 10.5.

The greatest reduction in pH following inoculation by bacteria was in the control THB group. This gives support and justification to the use of buffered solutions in order to reduce any decrease in the pH of experimental test agents once they are introduced into the flow cells.

3.4 Flow Cell Biofilm Growth Model

3.4.1 Control Flow Cell- Biofilm Grown in Unbuffered THB

Bacterial Viability Harvested From Dentine Discs

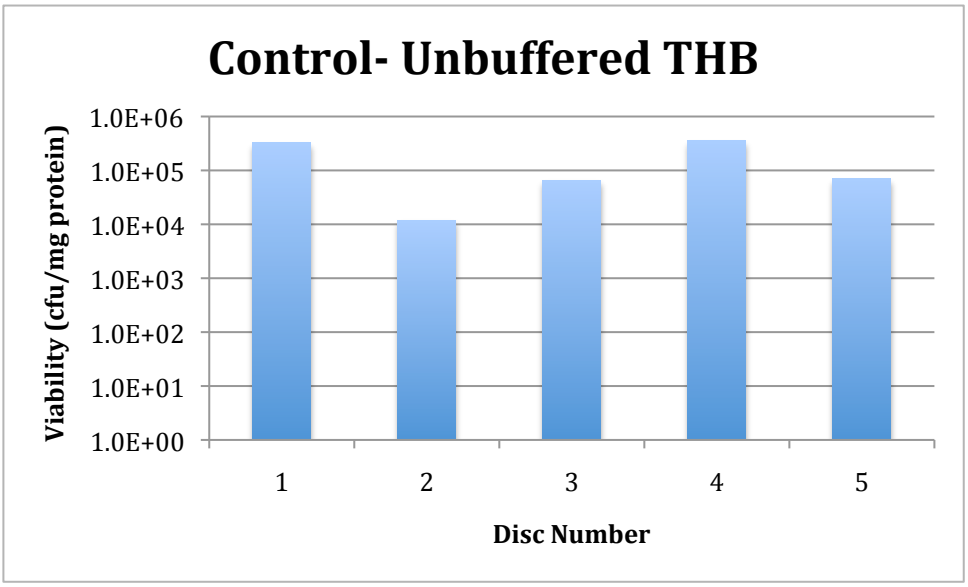


Figure 7: Bacterial viability from discs grown in the control flow cell following a 4 week growth phase and 1 week experimental phase in unbuffered THB

The viability counts per disc of the unbuffered, control flow cell were consistently in the range of 10^4 – 10^5 cfu/mg protein (Fig 7) .

SEM of Biofilm on Dentine Disc

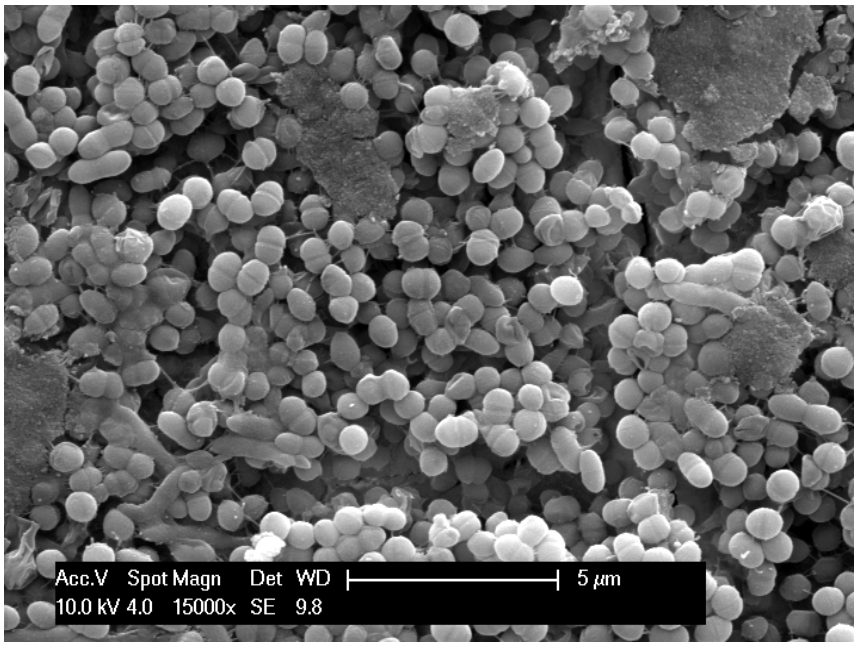


Figure 8: SEM image of the biofilm grown on a dentine disc in the control flow cell exposed to un-buffered THB (15,000 x magnification)

SEM images of the control specimen showed dense arrangements of bacteria confluent over the whole surface of the dentine disc specimens. Extra-cellular rope-like strands and often amorphous material appeared to join bacteria in aggregates (Fig 8).

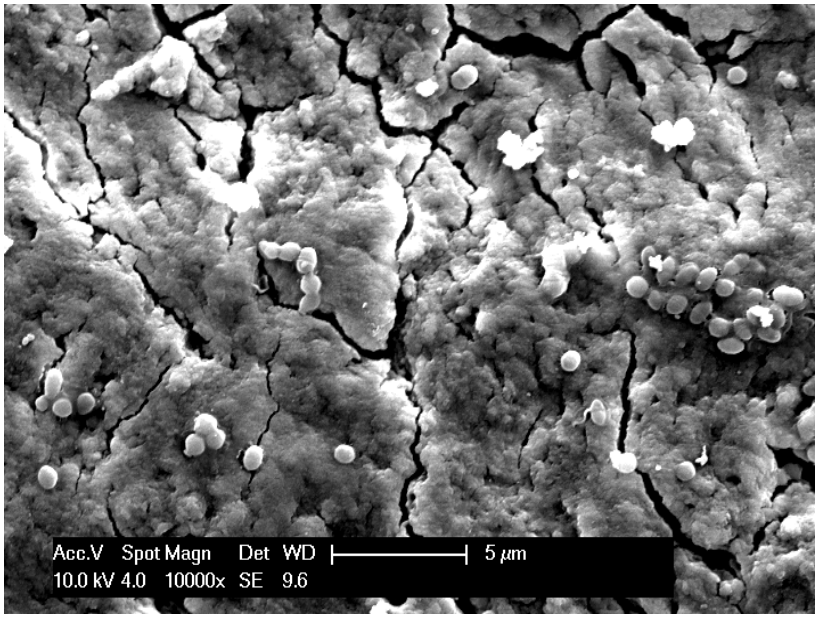


Figure 9: SEM image of the surface of a control disc after sonication dislodgement of the biofilm

The SEM in Figure 9, taken of a disc after sonication showed only a comparatively small number of bacterial cells remaining attached to the disc. This observation validated the biofilm detachment technique used to dislodge bacteria into saline for viability culture.

Confocal Imaging of Biofilm Stained with LIVE/DEAD™ BacLight® Stain

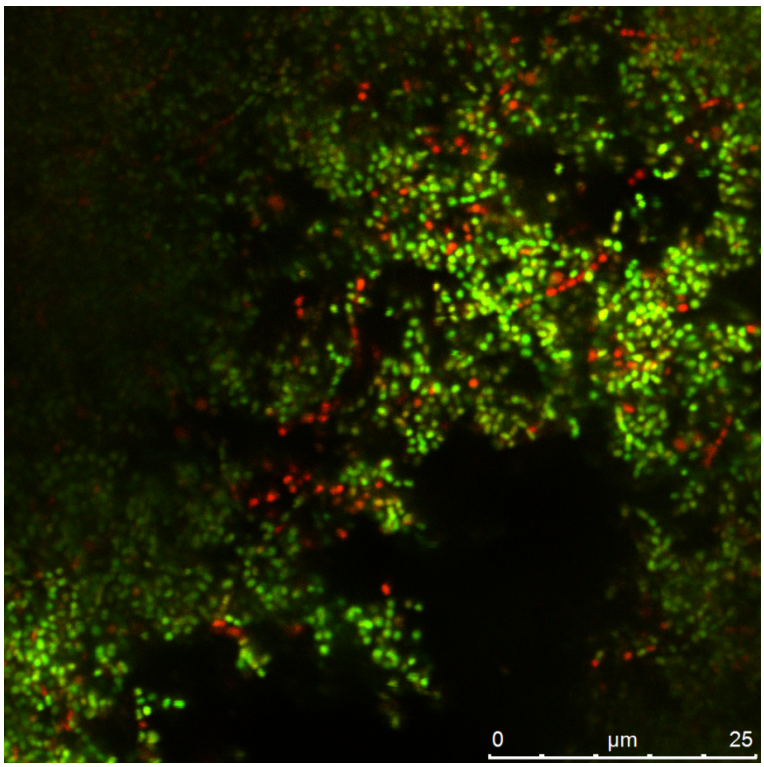


Figure 10: Confocal image of the biofilm grown on a dentine disc in the control flow cell exposed to unbuffered THB (x 63 objective lens). The biofilm has been stained with LIVE/DEAD™ BacLight® stain

LIVE/DEAD™ BacLight® viability staining in the control sample demonstrated a predominance of "viable", green, staining bacteria over "non viable", red, staining bacteria. The staining of cells in the control sample was distinctly either green or red (Fig 10).

3.4.2 Flow Cell 1- Rapid Increase in pH to 11.5

pH Stability of Buffered THB Media during the Experimental Phase

Day	pH of buffered THB media entering flow cell	pH of buffered THB media exiting flow cell
5	11.3	11.14
7	11.56	11.48

Table 7: Change in pH of buffered THB media entering and exiting the flow cell during the experimental phase when the flow cell was exposed to a rapid increase in pH to 11.5

pH of buffered THB and flow cell waste was not measured until day five of the experimental phase because in the rapid groups, the first three days of the experimental phase only entailed infusion of flow cells with unbuffered nutrient THB. On day four rapid infusion of buffered THB into the flow cell occurred, as detailed in the methodology (Section 2.7). The pH was only reduced slightly within the flow cell (Table 7). This was probably due to the presence of acidic end-products of bacterial metabolism as already mentioned in section 3.3.

Bacterial Viability of Harvested Biofilm Following a Rapid Increase to pH 11.5

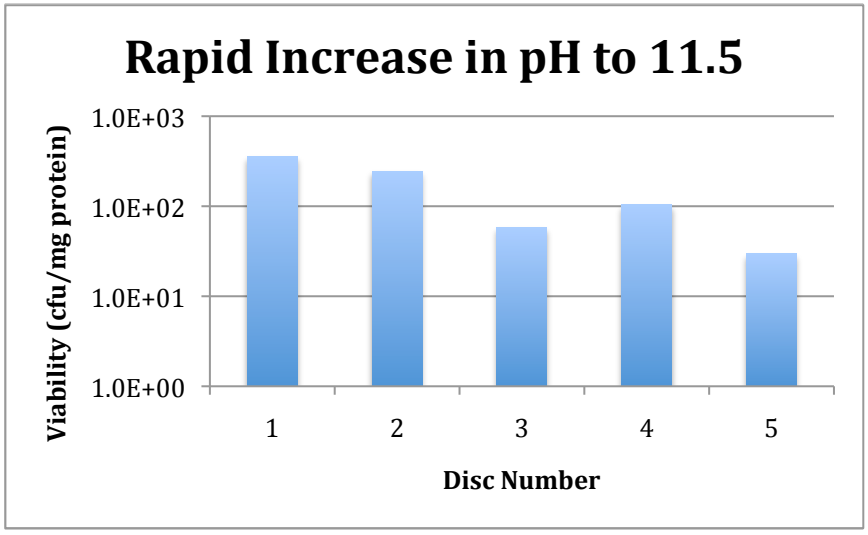
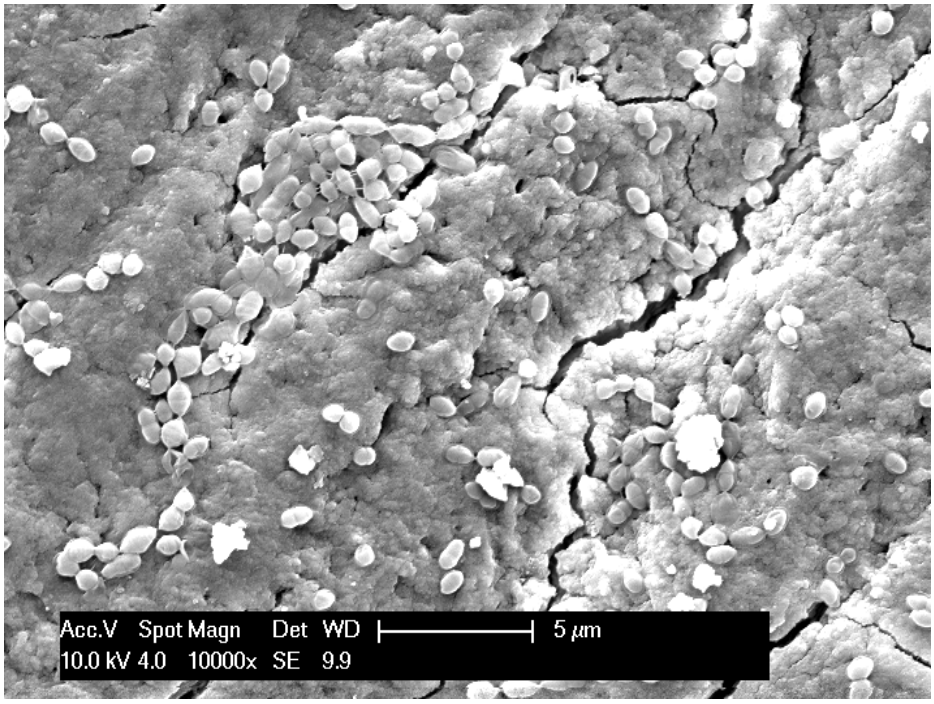


Figure 11: Bacterial viability of *E. faecalis* harvested from dentine discs after rapid increase in pH to 11.5 (cfu/mg protein)

Viability of the pH 11.5 group ranged between 10^1 – 10^2 cfu/mg protein (Fig 11). This viability represented >99.99% reduction in the viability of *E. faecalis* in comparison with the control group.

SEM of Biofilm on Dentine Disc Following Rapid Increase to pH 11.5

a.



b.

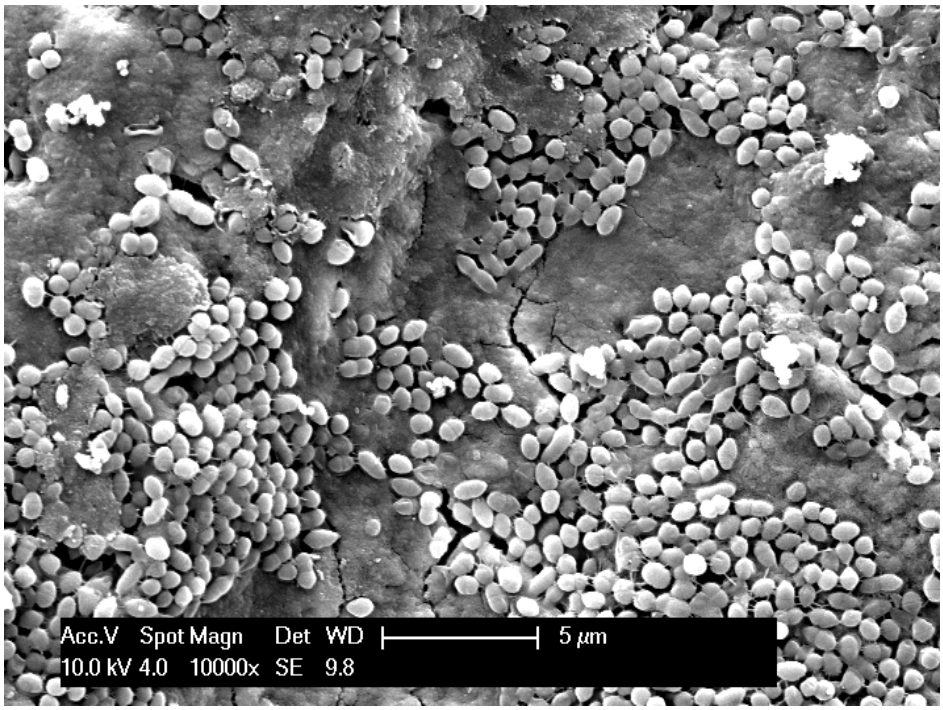


Figure 12: SEM images of the biofilm on dentine discs after rapid increase in pH to 11.5 (10000 x magnification)

SEM images of two discs shown in Figure 12 show a comparatively sparse scattering of bacteria compared to the control discs. The number of bacterial

cells visible in disc (b.) is greater than in disc (a.). This variation between discs mirrors the variation noted in the viability culture results.

Confocal Imaging of Biofilm Stained with LIVE/DEAD™ BacLight® Stain following Rapid Increase to pH 11.5

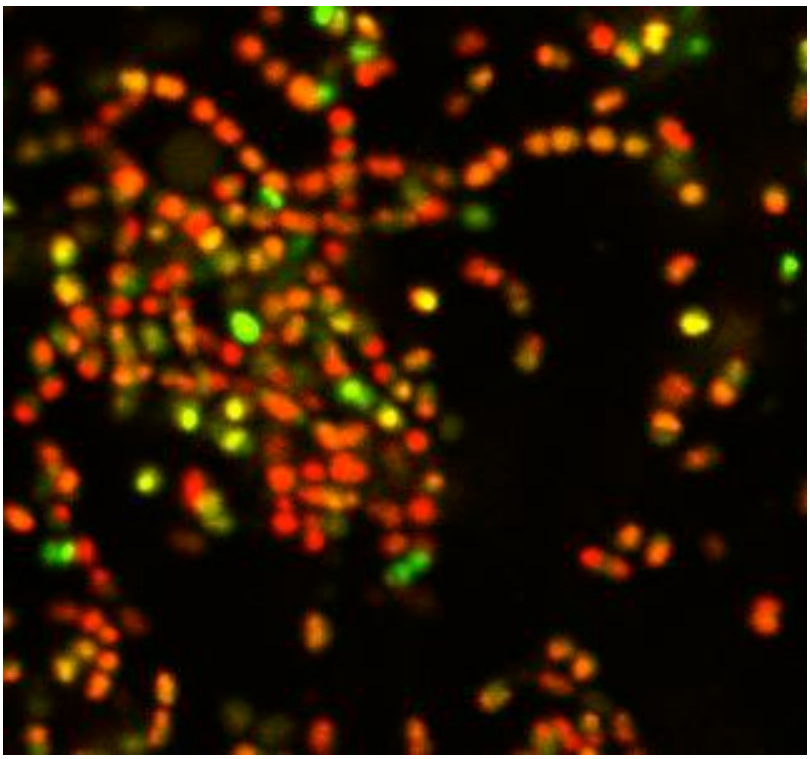


Figure 13: Confocal image of biofilms on a dentine disc after rapid increase in pH to 11.5 stained with the LIVE/DEAD™ BacLight® stain (x 63 objective lens).

In contrast to the control sample, the confocal image from the pH 11.5 rapid group showed a predominance of red, "dead", staining bacteria on the surface of the sample (Fig 13). While red staining bacteria were clearly more predominant both the red and green stains were less sharp and some cells appeared to have both some red and green staining or a yellow colouration.

3.4.3 Flow Cell 2- Slow Increase to pH 11.5

pH Stability of Buffered THB Media during the Experimental Phase

Day	pH of buffered THB media entering flow cell	pH of buffered THB media exiting flow cell
1	8.43	7.8
2	9.45	9.19
3	10.54	10.29
5	11.51	11.35
7	11.47	11.38

Table 8: Change in pH of buffered THB media entering and exiting the flow cell during the experimental phase when the flow cell was exposed to a slow increase in pH to 11.5

In this flow cell, buffered THB of increasing pH was introduced daily. Table 8 shows that the lower pH media (pH 8.43 and 9.45) showed a relatively large decrease in pH within the flow cells. However, as the pH of the buffered THB increased this differential with the flow cell pH decreased. This was probably due to decreasing biofilm viability and growth rate and thus less acidic metabolic end-products.

Bacterial Viability of Harvested Biofilm Following a Slow Increase to pH 11.5

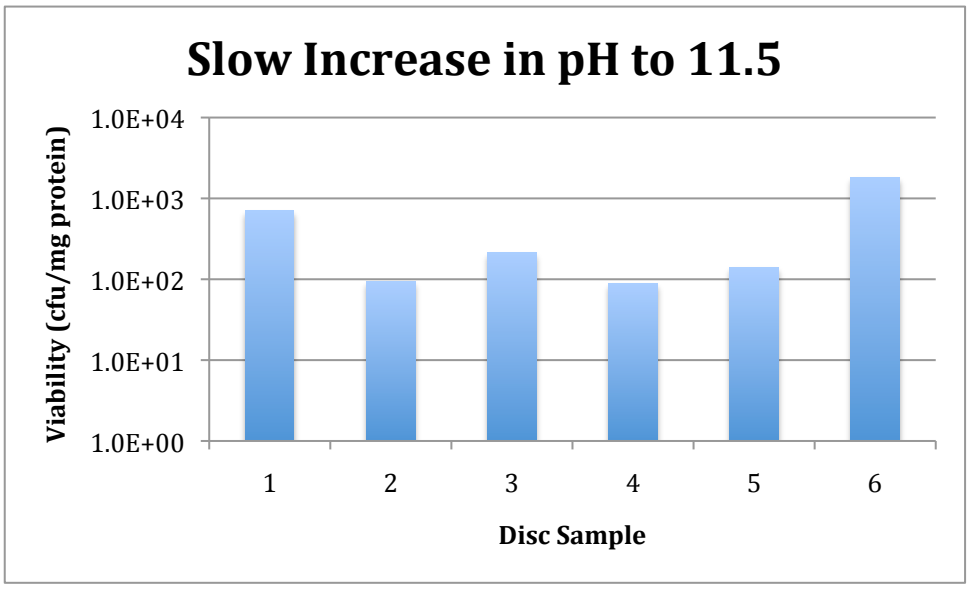
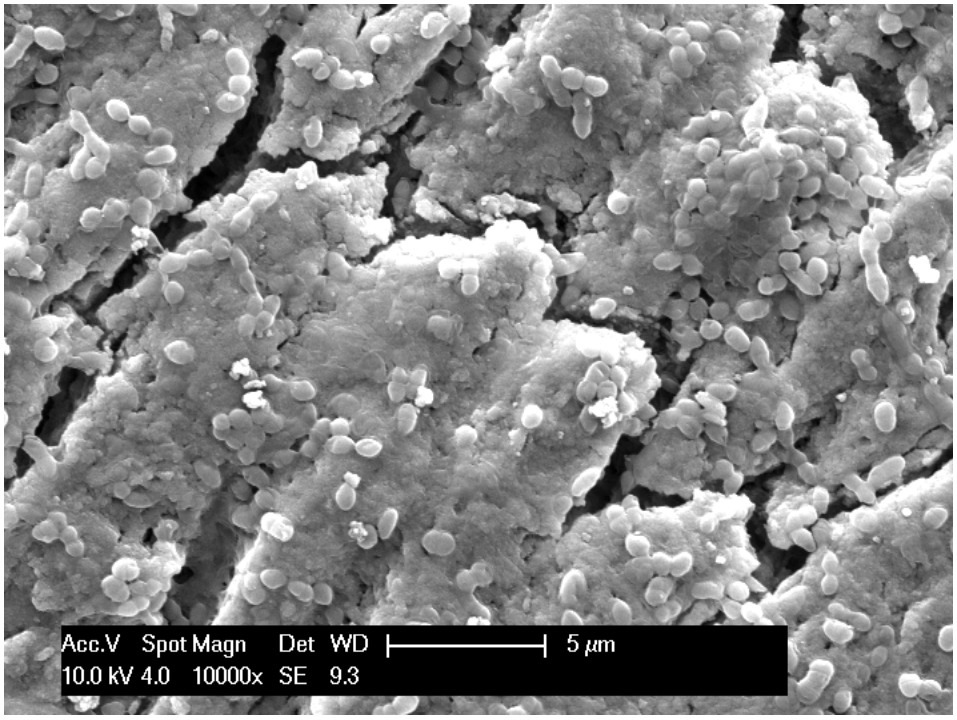


Figure 14: Bacterial viability of *E. faecalis* harvested from dentine discs after a slow increase in pH to 11.5 (cfu/mg protein)

Viability of the slow increase to pH 11.5 flow cell ranged between 10^1 – 10^2 cfu/mg protein (Fig 14). This viability represented >99.99% reduction in the viability *E. faecalis* in comparison with the control group.

SEM of Biofilm on Dentine Disc Following a Slow Increase to pH 11.5

a.



b.

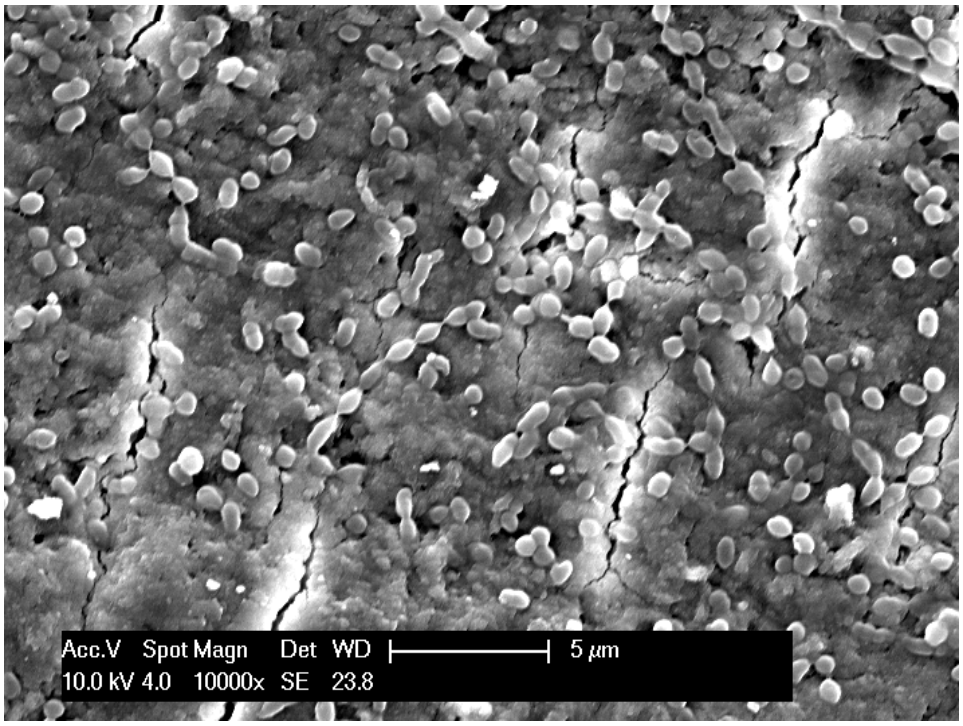


Figure 15: SEM images of the biofilm on dentine discs after a slow increase in pH to 11.5 (10000 x magnification)

The SEM images in Figure 15 represent replicate dentine discs taken from the flow cell which had the pH gradually increased from pH 8.1 to 11.5 over three days. Both show a sparse scattering of bacteria. Image (a.) shows one clump or aggregate of bacteria embedded in an amorphous extra-cellular material but mostly individual cells or short chains are apparent attached to the disc surface. The SEM images from this group are similar to the images in Fig 12 from pH 11.5 rapid pH rise group.

Confocal Imaging of Biofilm Stained with LIVE/DEAD™ BacLight® Stain Following a Slow Increase to pH 11.5

The confocal microscope was not working at the time this flow cell was processed and thus confocal microscopy could not be carried out for the slow increase to pH 11.5 flow cell. Confocal specimens cannot be stored for later examination because loss of bacterial viability may compromise *Bac*light staining, especially of live bacteria.

3.4.4 Flow Cell 3- Rapid Increase in pH to 12.5

pH Stability of Buffered THB Media during the Experimental Phase

Day	pH of buffered THB media entering flow cell	pH of buffered THB media exiting flow cell
5	12.58	12.4
7	12.5	12.4

Table 9: Change in pH of buffered THB media entering and exiting the flow cell during the experimental phase when the flow cell was exposed to a rapid increase in pH to 12.5

The pH of the buffered THB was set at 12.5 and a pH range of 12.58 – 12.5 was observed. Only a small pH reduction of 0.1 - 0.18 was seen when the nutrient was pumped into the flow cell (Table 9).

Bacterial Viability of Harvested Biofilm Following a Rapid Increase in pH to 12.5

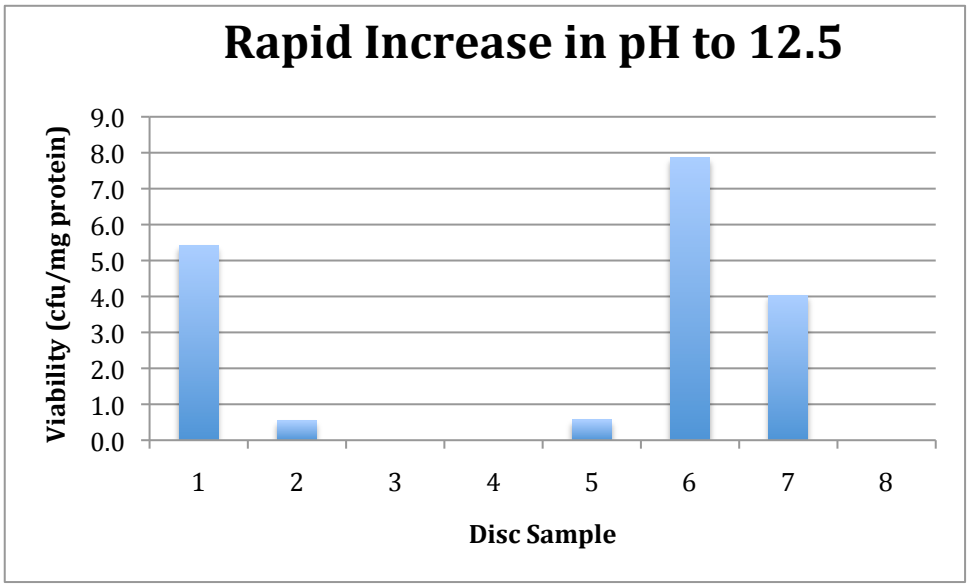
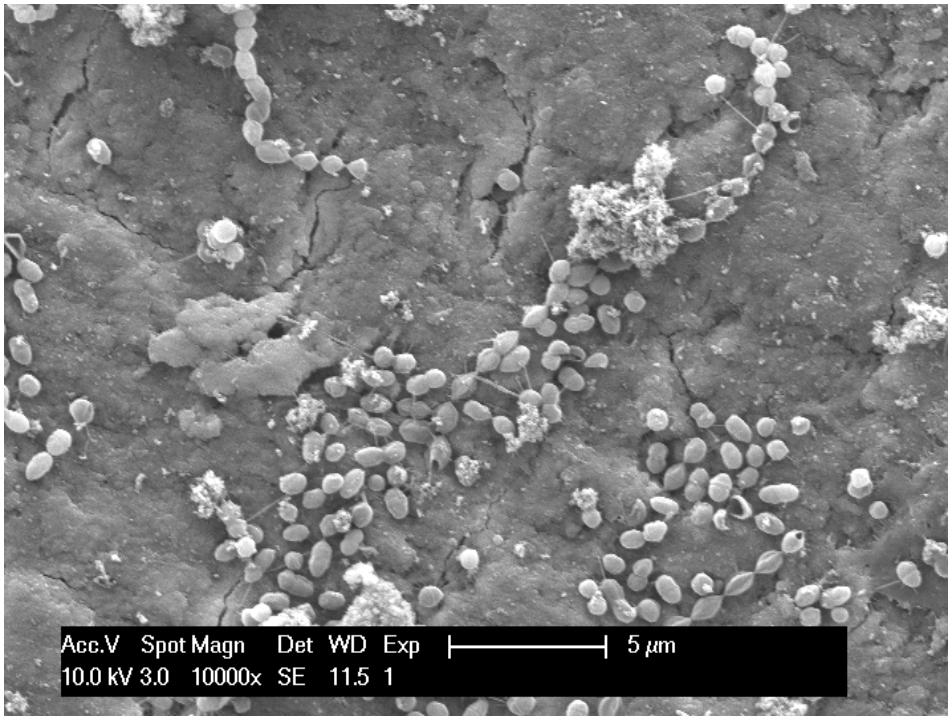


Figure 16: Bacterial viability of *E. faecalis* harvested from dentine discs after a rapid increase in pH to 12.5 (cfu/mg protein)

Figure 16 shows viable bacterial growth from only five disc samples. Three disc samples had no cultivable bacteria detected. This viability represented a >99.99% reduction in the viability of *E. faecalis* in comparison with the control group.

SEM of Biofilm on Dentine Disc Following Rapid Increase to pH 12.5

a.



b.

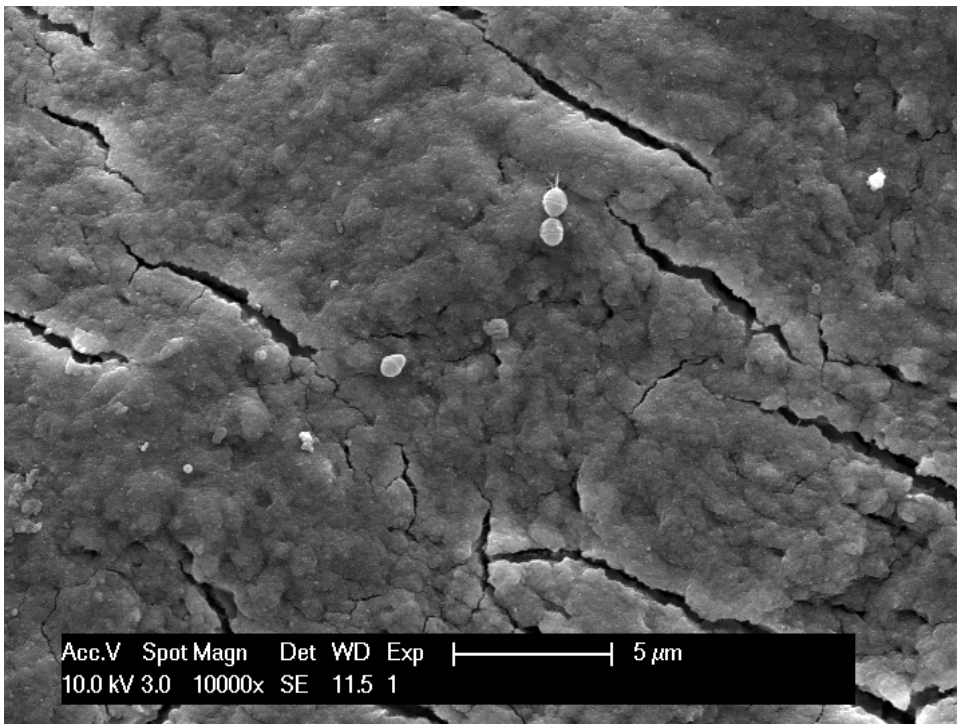


Figure 17: SEM images of the biofilm on dentine discs after a rapid increase in pH to 12.5 (10000 x magnification)

SEM images of two replicate discs seen in Figure 17 show a sparse scattering of *E. faecalis* compared to the previous pH 11.5 group. Bacteria appear well anchored to the dentine surface with fibrous-like structures. In (b.) the bacteria seen were the only cells found despite extensive searching over the entire disc. This variation between discs seems to match the variability of the viability count although the appearance of cells with SEM imaging does not necessarily mean they are viable.

Confocal Imaging of Biofilm Stained with LIVE/DEAD™ BacLight® Stain Following Rapid Increase to pH 12.5

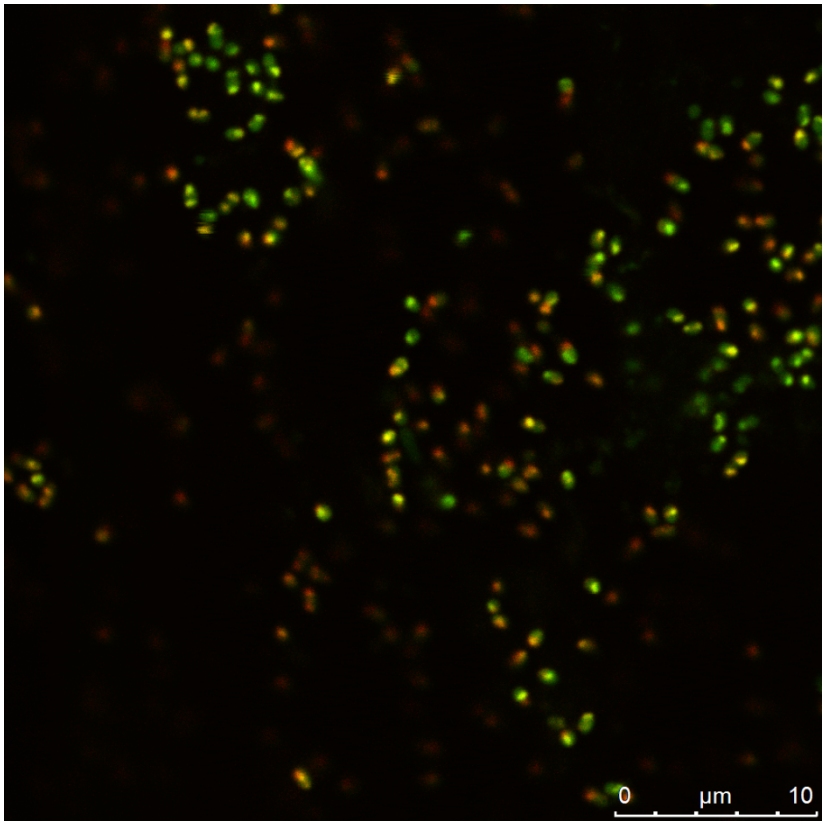


Figure 18: Confocal image of biofilms on a dentine disc after a rapid increase in pH to 12.5 stained with the LIVE/DEAD™ BacLight® stain (x 63 objective lens).

Confocal microscopy revealed fewer bacteria on the dentine discs compared to the control group. Of note in Figure 18 is the presence of yellow staining cells and other cells that appear to have both some distinct red and green stain in them. This is in contrast to control and pH 11.5 confocal images which had more

distinctly green or red staining bacteria (Figs 10 and 13). This unusual staining makes it difficult to definitively say whether individual cells were viable or non-viable.

3.4.5 Flow Cell 4- Slow Increase in pH to 12.5

Stability pH of Buffered THB Media During the Experimental Phase

Day	pH of buffered THB media entering flow cell	pH of buffered THB media exiting flow cell
1	9.3	8.97
2	10.33	10.04
3	12.09	11.48
5	12.49	12.4
7	12.4	12.3

Table 10: Change in pH of buffered THB media entering and exiting the flow cell during the experimental phase when the flow cell was exposed to a slow increase in pH to 12.5

A similar small reduction in pH occurred to the buffered THB media in this group similar to the pH 11.5 slow increase group (Table 10). Greater reductions in pH occurred for the lower pH buffered THB but this reduction decreased for the higher pH media. On day 3, buffered THB with pH 11.5 +/- 0.2 was introduced to the flow cell. For an unknown reason, this media measured 12.09 when its pH was measured again at the end of its use. This was outside the target range of pH 11.5 +/- 0.2 (Section 2.4). However, the flow cell pH was 11.48 which was very close to the target pH and thus this flow cell was continued and included in the results.

Bacterial Viability of Harvested Biofilm Following Slow Increase to pH 12.5

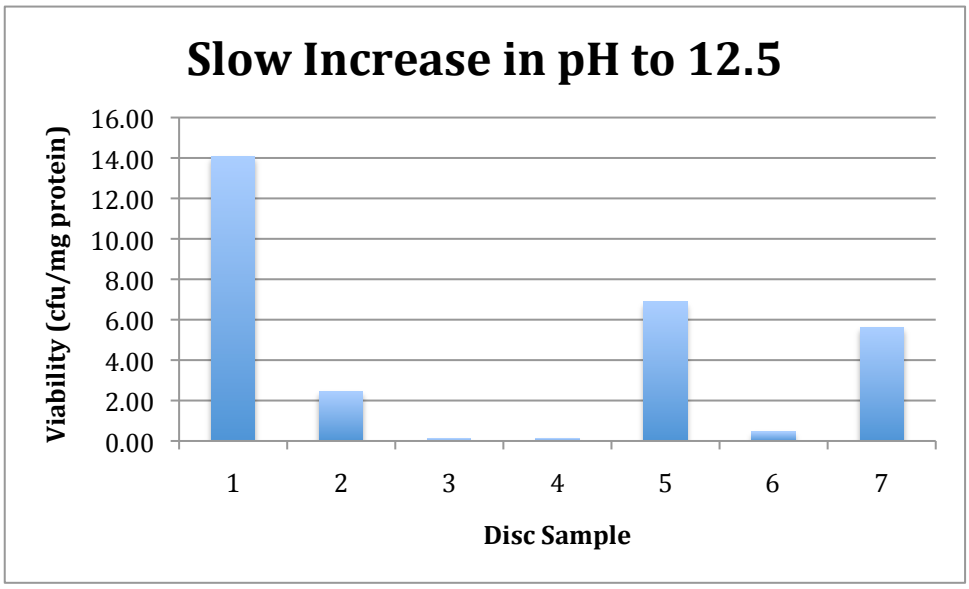


Figure 19: Bacterial viability of *E. faecalis* harvested from dentine discs after a slow increase in pH to 12.5 (cfu/mg protein)

Figure 19 shows viable bacterial growth from all seven disc samples. This viability represented a 99.99% reduction in the viability of *E. faecalis* in comparison with the control group. Viability from the pH 12.5 rapid and slow groups appear very similar.

SEM of Biofilm on Dentine Disc Following Slow Increase in pH to 12.5

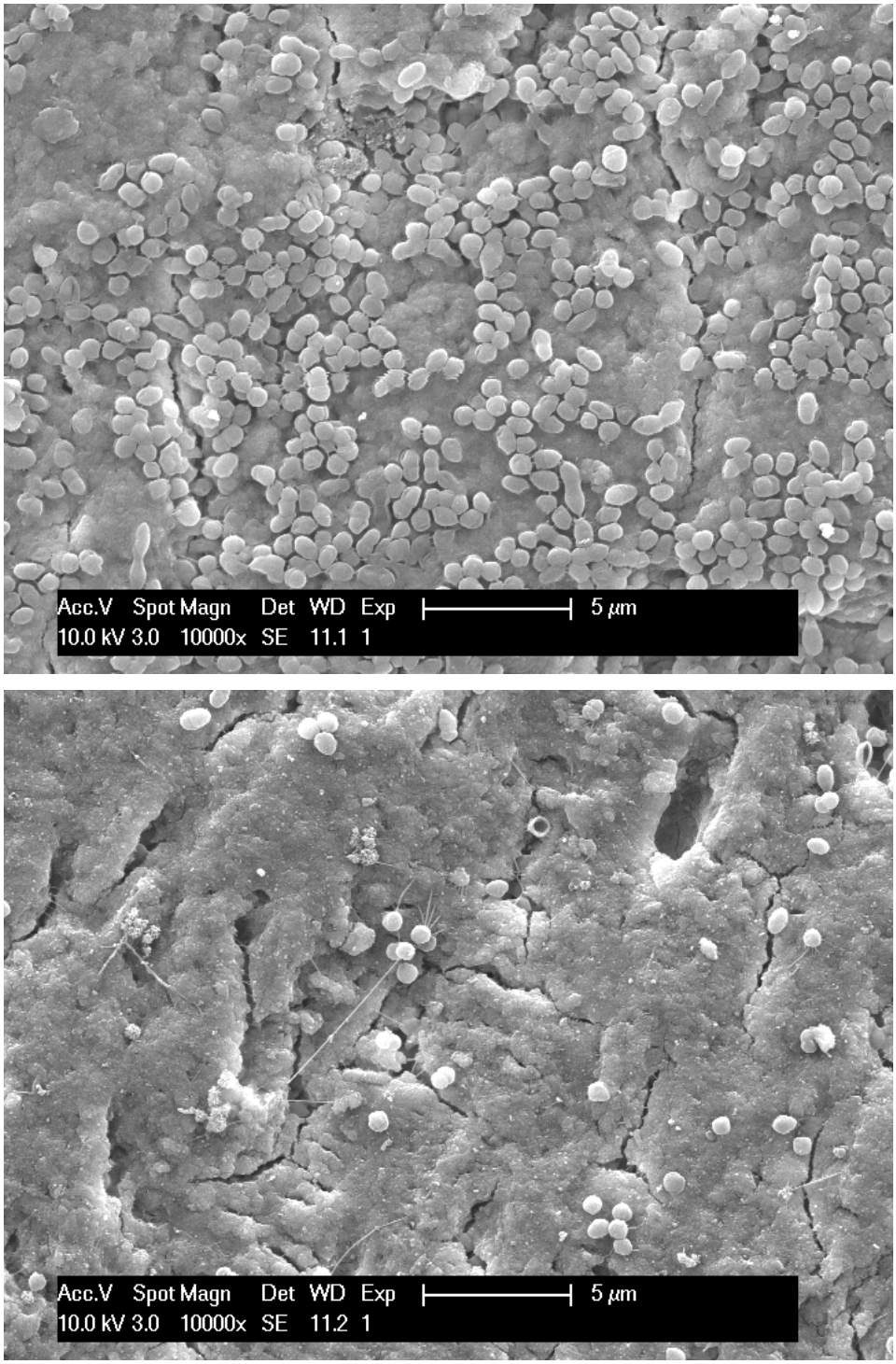


Figure 20: SEM images of the biofilm on dentine discs after a slow increase in exposure to pH 12.5 (10000 x magnification)

The SEM images in Figure 20 are broadly similar to the pH 12.5 rapid increase group. Similar variability between two different discs, (a.) and (b.), is seen but again this matches the variability seen between discs in the viability cultures.

Confocal Imaging of Biofilm Stained with LIVE/DEAD™ BacLight® Stain Following Slow Increase in pH to 12.5

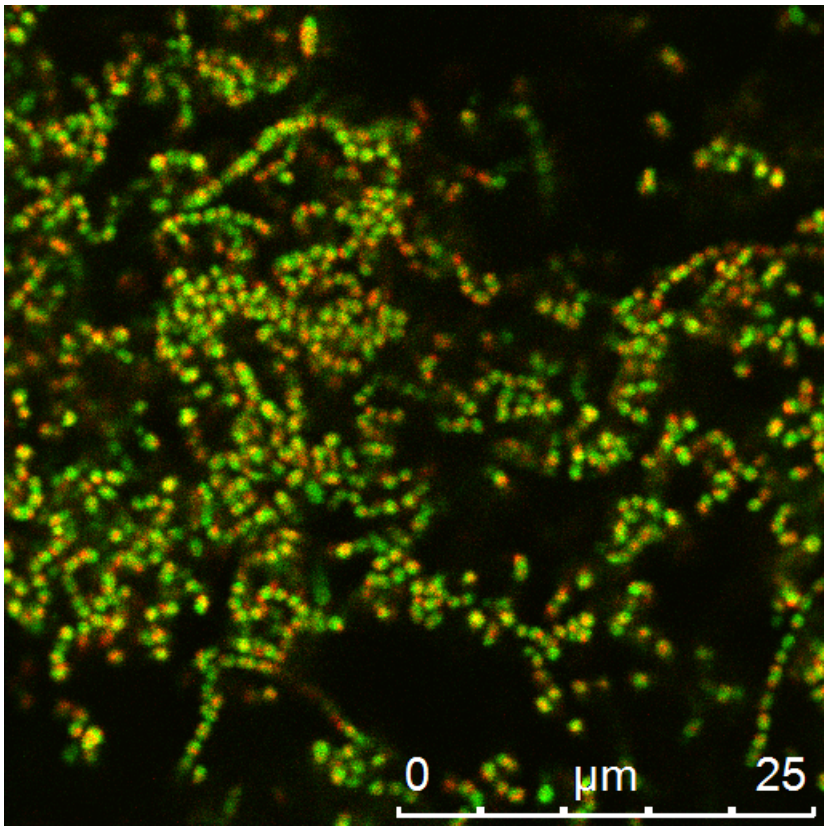


Figure 21: Confocal image of biofilms on a dentine disc after slow increase in exposure to pH 12.5 stained with the LIVE/DEAD™ BacLight® stain (x 63 objective lens).

The confocal images taken in this group, represented by Figure 21, show greater numbers of cells compared to confocal images taken from the pH 12.5 rapid group. However, as in the 12.5 rapid group (Figure 18), the appearance of yellow cells and cells with both green and red stain make determining live or dead status difficult.

3.5 Comparison of Bacterial Viabilities Between Different Flow Cell Protocols

3.5.1 Comparison of Rapid versus Slow Increases in pH

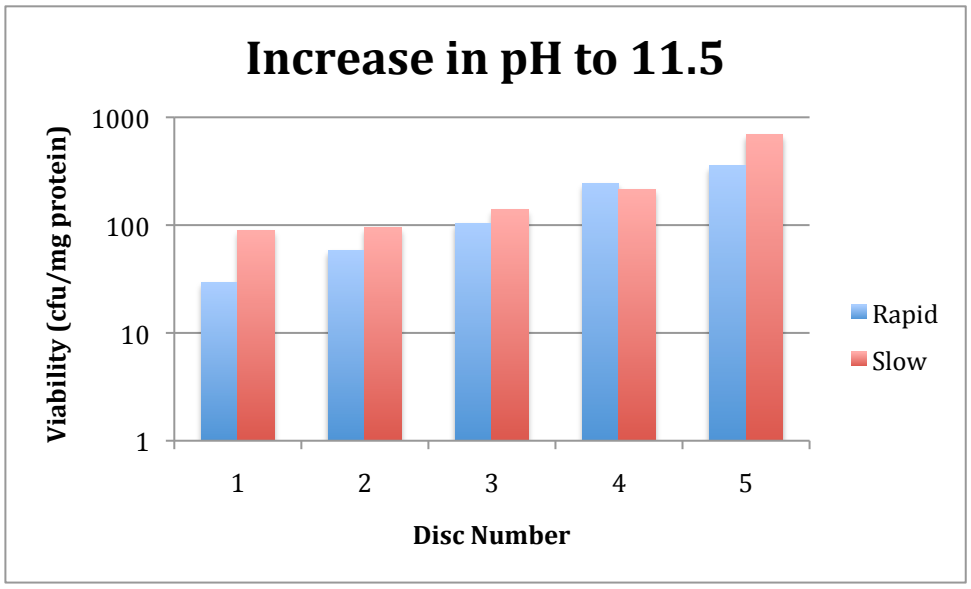


Figure 22: Comparison of bacterial viability on individual disc samples between flow cells with rapid and slow increases in pH to 11.5. Discs were ranked from lowest to highest viability (cfu/mg protein)

Individual disc sample viabilities were ranked highest to lowest for both the pH 11.5 rapid and slow flow cells. When these were directly compared, as in Figure 22, there appeared to be no significant difference between the flow cells with rapid and slow increases in pH to11.5. Statistical analysis was carried out by applying a nonparametric Wilcoxon test. No statistically significance difference was seen between the rapid and slow groups ($p = 0.43$).

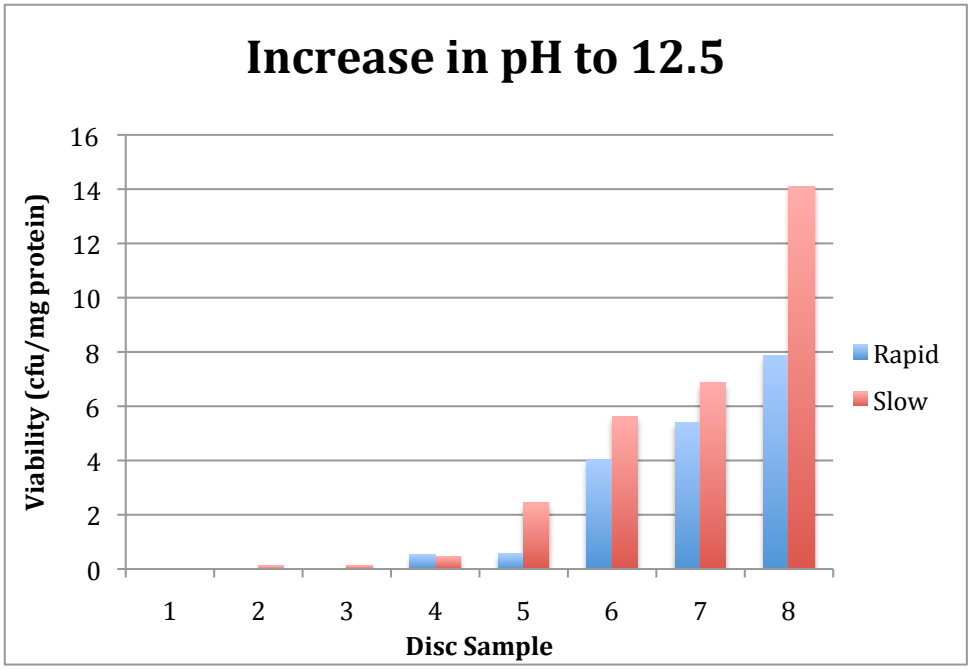


Figure 23: Comparison of bacterial viability on individual disc samples between flow cells with rapid and slow increases in pH to 12.5. Discs were ranked from lowest to highest viability (cfu/mg protein)

When the highest to lowest disc sample viabilities were directly compared (Figure 23), there was also no significant difference between the flow cells with rapid and slow increases to pH 12.5. Statistical analysis was carried out by applying a nonparametric Wilcoxon test ($p = 0.4$).

3.5.2 Comparison of Rapid versus Slow Increase to pH 12.5 when 3 Discs Were Used per Sample

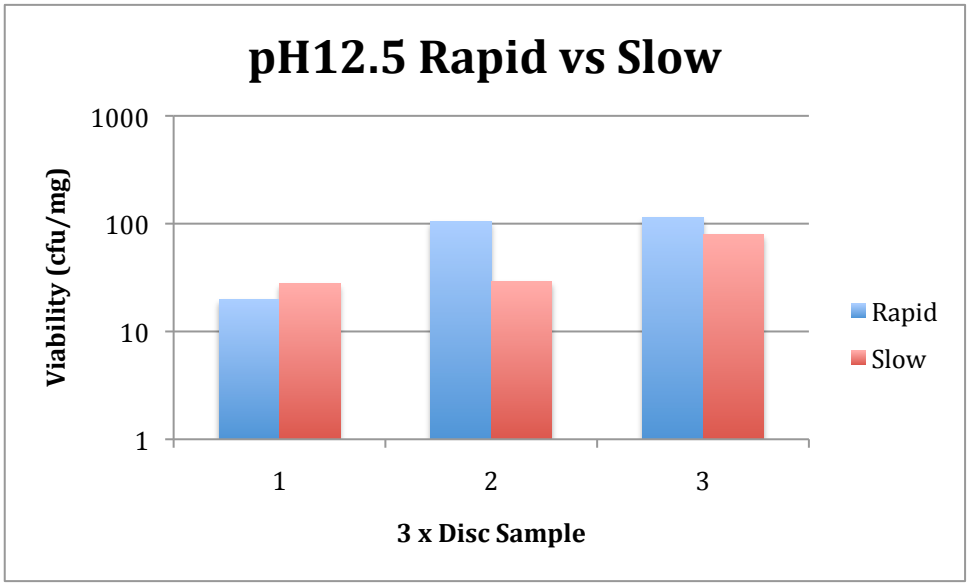


Figure 24: Comparison of bacterial viability between flow cells with rapid and slow increases in pH to 12.5. Each sample included the viable bacteria from three discs (cfu/mg protein)

After the experimental phase of the replicate experiment increasing the pH slowly or rapidly to maximum pH 12.5, three discs were combined per sample instead of one disc per sample (Section 2.8.1). This reduced the number of replicate samples per flow cell but aimed to increase the viable cell numbers per sample. However, the results seen in Figure 24 show no apparent difference between the rapid and slow groups similar to Figures 22 and 23. The sample size for this experiment was too small to carry out statistical analysis.

3.5.3 pH 11.5 vs pH 12.5

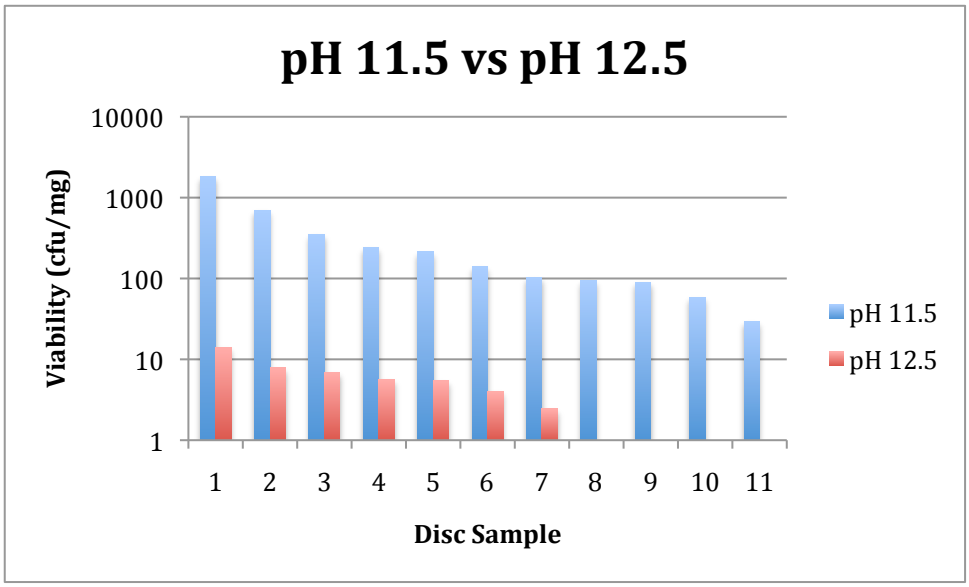


Figure 25: pH 11.5 vs pH 12.5 viability

Given that no significant difference existed between the rapid and slowly increasing groups for either maximum pH 11.5 or pH 12.5, all samples were grouped together. A comparison of bacterial viability following exposure to pH 11.5 or pH 12.5 was made. There was a statistically significant increase in bacterial viability from those samples exposed to a maximum pH of 11.5 compared to those exposed to pH 12.5 (Wilcoxon test ($p = 0.0003$)).

4. Discussion

4.1 Dentine Disc Serial Transfer Biofilm Growth Model

Initial experiments for this study tested a biofilm growth model that entailed serial transfer of dentine discs every 24 hours to fresh media. Over the course of four weeks, SEM analysis showed that the biofilm on the discs increased and produced a uniform covering of bacteria on the dentine disc surface. It was not a particularly dense arrangement of bacteria. If the discs were left for several weeks longer then possibly a more confluent biofilm may have developed. However, experimental practicalities needed to be considered and made this model unsuitable. The other issue with this initial biofilm model was that serial transfer of discs every 48 hrs into fresh media allowed regular chances for contamination to occur.

A flow cell model had previously been developed by our group which has been shown to produce confluent biofilms over a four-week period and so it was decided to utilize this model (Plutzer, 2009).

4.2 Flow Cell Biofilm Growth Model

SEM images of the disc samples from the control flow cell revealed a dense confluent biofilm over the entire disc. It was thus decided to use this model to compare the survival of an *Enterococcus faecalis* biofilm when exposed to rapid or slow increases in external pH.

The maximum pH used initially was 11.5. This pH was chosen because it has previously been demonstrated that pH 11.5 does not support growth of *E. faecalis* in a planktonic state (Evans *et al*., 2002, McHugh *et al*., 2004). It was expected that a biofilm would provide a degree of protection but a large reduction in viable bacteria could still be expected due to the longer length of exposure. The one week duration of the experimental phase of treatment was based on an *in vivo* study using culture techniques by Sjögren *et al.* (1991) which demonstrated the elimination of bacteria from all canals sampled following

application of calcium hydroxide for one week. While large reductions (99.99%) of viable bacteria were seen when exposed to pH 11.5, overall, viable bacteria were not eliminated completely. There was no statistically significant difference in viable counts between rapid or slow increases in pH. As a result it was decided to increase the maximum pH to 12.5 to better elucidate any difference between the rapid and slow groups. An increase to pH 12.5 is also more clinically relevant because this is the pH of calcium hydroxide (Siqueira and Lopes, 1999). This increase in maximum pH significantly reduced *E. faecalis* viability further but did not completely eliminate bacteria from all samples in either group. Once again, no statistical difference was seen between the rapid and slow groups.

4.3 Bacterial Reduction

The first finding from this study was that *E. faecalis* is susceptible to high pH. When compared with the control, the introduction of high external pH resulted in a highly significant decrease in viable bacteria. A reduction of over 99% in viable bacteria was achieved regardless of whether the maximum pH was 11.5 or 12.5 or whether the pH was increased rapidly or slowly. This finding is in partial agreement with previous studies which examined the effect of different pH on planktonically grown *E. faecalis*. Byström *et al.* (1985) found viability of planktonic bacteria were reduced by over 99% when exposed to a calcium hydroxide solution at pH 12.5 within 30 min. However, at pH 11.5 they did not show as great a reduction as in this study, although the time course of their contact time was only 24 hrs, whereas in this study the maximum pH of 11.5 was maintained for four days (Byström *et al*., 1985). Longer exposures to alkaline conditions are more clinically relevant because clinically calcium hydroxide is normally placed from 1-4 weeks (Byström *et al*., 1985, Shuping *et al*., 2000, Sjögren *et al*., 1991). In contrast, Evans *et al.* (2002) had similar reductions of greater than 99% when *E. faecalis* suspensions were exposed to pH 11.1 and 11.5 for even shorter durations of 15 and 30 min. McHugh *et al.* (2004) specifically looked at the pH and time required to completely eliminate viable bacteria from a planktonic culture of *E. faecalis*. At pH 11.5 and 12.5 this was achieved in 24 hours.

The differences in time-courses as well as the use of planktonic cultures of *E. faecalis* in these studies make definitive comparisons with the present study difficult. However, given the increasing acceptance of biofilms as a crucial mechanism of infection within the root canal, comparisons with historical planktonic work are interesting. It could be suggested by comparing this study with that of McHugh *et al.* (2004) that biofilms require a longer and higher pH exposure than planktonic bacteria to achieve a 100% killing efficacy. This result would concur with previous findings that bacteria within biofilms have a greater resistance to antimicrobial agents than their planktonic counterparts (Brandle *et al*., 2008, Chávez de Paz *et al*., 2007, Svensåter and Bergenholtz, 2004). This finding is not surprising given the protections afforded by biofilms. However, the heterogeneity between studies makes this finding only suggestive and not definitive. Further direct comparisons between biofilm and planktonic bacteria in the endodontic context would be of interest.

4.4 Bacterial Survival

While there was a very significant reduction in the total viable bacterial numbers after pH exposure of 12.5, some bacteria still survived. Although the numbers were very small they may still be of clinical significance because the presence of cultivable bacteria within root canals prior to obturation has been shown to reduce success rates for those teeth (Molander *et al*., 2007, Ng *et al*., 2008, Sjögren *et al*., 1997). In fact, Sundqvist *et al.* (1998) found in an *in vivo* study that the recovery of *E. faecalis* immediately prior to obturation in cases undergoing retreatment, resulted in a lower success than teeth testing positive for other bacteria. Therefore, the fact that the present study showed persistence of *E. faecalis* in the presence of a high pH over a clinically relevant time-course, analogous to the use of calcium hydroxide *in vivo*, may be of concern clinically especially for retreatment cases. Having said this, the recoverable bacteria in this study were very low in number and it is currently unknown what degree of persistent microbial numbers are required to maintain or develop apical periodontitis.

Although it is generally accepted that achieving a truly sterile canal is an unlikely outcome following chemo-mechanical debridement of infected root canals, the concept of entombment with obturation is often thought to deprive residual bacteria of nutrients and thus lead to their eventual death. However, the residual viable *E. faecalis* seen in this study would be of particular concern clinically because this bacterium has been shown to persist over long periods in a low nutrient environment (Figdor *et al*., 2003, Sedgley *et al*., 2005d). These studies have demonstrated the ability of *E. faecalis* to survive long periods of starvation and still be able to recover once nutrient becomes available again. If coronal or apical leakage were to occur, even small numbers of surviving *E. faecalis* may once again flourish and become a source of persistent apical periodontitis.

The reasons why some *E. faecalis* survived high pH exposure in the present study can only be speculated upon. Two potential possibilities include changes in gene expression that results in an alkaline resistant phenotype or alternatively, alkaline pH has selected for bacteria with genotypic differences. Genotypic variations may have occurred over the course of the four-week growth phase producing random genetic mutations that could have conferred resistance to high pH. However perhaps a more likely explanation is that a phenotypic adaptation occurred in response to pH stress by some bacteria. Why this may have been expressed by some *E. faecalis* and not others could have been due to their distribution within the biofilm.

Phenotypic changes may be induced by changes in the external environment such as a rise in pH. An example may be the induction of stress response mechanisms (Flahaut *et al*., 1997, Kayaoglu and Ørstavik, 2004). These stress responses may be specific to the type of challenge or they may be general stress response mechanisms. Stress responses that are of a general nature may induce a phenotype that is more resistant to antimicrobial challenge. For example, the response of *E. faecalis* to an alkaline pH has been demonstrated to result in cross-protection against bile salt insults (Flahaut *et al*., 1997). If this were the case, the surviving bacteria in this study may represent a more resistant group of bacteria that are being selected for by an increase in pH. Such selection of potentially more virulent bacteria by the use of a high pH medicament such as

calcium hydroxide has been raised as a possible cause for concern in root canal therapy (Chávez de Paz *et al*., 2007, Hancock, 2001). Selection of resistant bacteria may make their elimination with further endodontic therapy even more difficult. This may possibly explain why lower success rates are seen with retreatment therapy than with primary endodontic treatment (Sjögren *et al*., 1990, Sundqvist *et al*., 1998).

The term persister cells has been coined to describe another phenotypic variation. It has been suggested that amongst populations of genetically identical bacteria, persister phenotypes randomly develop. Persisters are thought to be tolerant of antimicrobial challenges in that while they do not divide and grow while under the stress of an insult they do remain viable (Lewis, 2007). Persisters are typically discussed in respect to antibiotic tolerance as opposed to challenges such as high pH. However, they are commonly thought to occur in biofilms and possibly are a key factor in bacterial survival in biofilm infections (Lewis, 2007). The persister phenomenon may explain the survival of bacteria following the high pH shift used in this study. Such persister bacteria are not thought to have ongoing resistance because once an antimicrobial challenge has passed they have been shown to replicate and produce offspring demonstrating normal susceptibility to the same challenge (Lewis, 2007). In this respect, if the surviving bacteria were a result of the persister phenomenon their ongoing susceptibility to conventional endodontic therapy will not change. Thus, treatment with calcium hydroxide would not be creating phenotypically more resistant bacteria. Possible future research investigating the possible persister nature of the surviving bacteria from this study is discussed in Section 4.10.

4.5 Rapid versus Slow Increase in pH

The primary aim of this study was to establish whether a slow increase in pH would result in a greater survival of *E. faecalis* within a biofilm when compared with a rapid increase in pH over a clinically relevant time period. Such an outcome could occur as a result of having more time to adapt to a slowly increasing pH prior to reaching a normally lethal pH level. Such a slow increase in pH might be experienced in the outer aspects of dentinal tubules or accessory

canals where increases in pH following calcium hydroxide application are dependant on diffusion. However, statistical analysis of the results of *E. faecalis* viability testing in this study showed no significant difference between the rapid and slowly increasing pH groups.

This finding has clinical relevance to root canal medication with calcium hydroxide. Calcium hydroxide has two main pharmacokinetic limiting factors within the root canal. Firstly, it is slow to diffuse through the root canal system and dentine due to its poor solubility and potential buffering by dentine (Haapasalo *et al*., 2000). Secondly, because of these factors, it may not necessarily result in a rise to pH 12.5 throughout the entire root canal system (Nerwich *et al*., 1993). Within the main root canal, where calcium hydroxide can be directly delivered, a rapid and large rise in pH to that of calcium hydroxide can be expected. Other areas, such as the peripheral dentine are unlikely to reach the same pH or will only increase in pH slowly.

The present study attempted to create a clinically relevant simulation. We used a slow but gradually increasing rise in pH from sub-lethal levels to normally lethal levels to mimic calcium hydroxide dressing *in vivo*. Clinically, such a slow rise due to diffusion and buffering may occur over several days or weeks until a maximal pH is eventually reached. The one week duration of the experimental phase is clinically relevant, as described in Section 4.2. The three days of a gradual increase in pH followed by a sustained four day phase at maximum pH represents an arbitrary timeframe that we took to represent the pH change at a point somewhere within the pulp-dentine complex. The location where such a pH change occurs is most likely within dentinal tubules close to the main root canal, lateral or accessory canals or other areas of the complex root canal system beyond the reach of mechanical instrumentation and irrigation. In these areas a slow rise in pH can be expected following placement of medicament in the nearby main canal because of diffusion and buffering factors. However, giving a precise location as to where in a natural tooth our experimental procedure represents is not possible.

Other studies have also investigated a potential adaptation of *E. faecalis* to high pH. Similar to the initial slow rise in pH used in this study, Evans *et al.* (2002) and Flahaut *et al.* (1997) exposed *E. faecalis* to sub-lethal pH prior to an exposure to a normally lethal pH. The results of this study concur with those of Evans *et al.* (2002) who found no apparent adaptive response or increase in survival when planktonic *E. faecalis* was exposed to a sub-lethal pH of 10.3 before exposure to pH 11.5. In contrast, the results of this study do not reflect those of Flahaut *et al.* (1997) who demonstrated a greater survival of *E. faecalis* when a planktonic culture was exposed to pH 10.5 prior to exposure to pH 11.9. However, the methodologies used by the studies are very disparate. Neither Flahaut *et al.* (1997) nor Evans *et al.* (2002) attempted to simulate root canal dressing with calcium hydroxide. Both used planktonic bacteria whereas it is now well accepted that bacteria within the root canal form and exist primarily as more resistant biofilms. Calcium hydroxide dressing is also normally recommended to be placed for a minimum of one week whereas the studies in question were only carried out for 30 min. They used single, short exposures to a sub-lethal pH as opposed to this studies continuous, slowly increasing rise in pH which more closely resembles the *in vivo* situation.

Calcium hydroxide exerts its antibacterial action by causing a local rise in pH (Siqueira and Lopes, 1999). The results of some recent studies have questioned the efficacy of calcium hydroxide as a medicament (Peters *et al*., 2002, Sathorn *et al*., 2005). From a clinical perspective, while there may be a question over efficacy, as long as no harm is done by application of calcium hydroxide, the decision to use it or not can reasonably be left to the practitioner. However, some concern has been raised that calcium hydroxide may be harmful by selecting for or inducing more resistant bacteria (Chávez de Paz, 2007, Hancock, 2001). However, the results of this study showed no difference in survival between *E. faecalis* biofilm exposed to a rapid or slow increases in pH. Given this study aimed to simulate conditions within the root canal, this result would suggest no difference in the adaptive ability of *E. faecalis* if it were located, for example, down an accessory canal where a slow rise in pH is more likely or whether it was located in the main root canal where a rapid rise in pH would be more likely.

Given that slow diffusion is considered a limitation of calcium hydroxide, this study would suggest that this limitation may not be significant.

4.6 pH 11.5 vs pH 12.5

Most endodontic bacteria are likely to be sensitive to rises in pH (Byström *et al.*, 1985). However, an incomplete elimination of bacteria due to an inadequate rise in pH may lead to a residual infection of more innately pH resistant bacteria. The resultant residual infection may make elimination by retreatment procedures more difficult especially if calcium hydroxide was to be relied upon again. While the aim of this study was not to specifically look at the bactericidal effect of different alkaline pHs, comment can be made with respect to this. Significantly more bacteria survived when the maximum pH was 11.5 compared with 12.5 (Wilcoxon test ($p=0.0003$)). This finding concurs with Bystrom *et al.* (1985). Thus, in order to prevent a large residual presence of *E. faecalis*, a pH as high as possible is necessary and the medicament given sufficient time to reach its maximum pH throughout the canal system.

It is possible that the few surviving bacteria in this study did not reach the maximum pH within the flow cell due to biofilm protection. This seems doubtful when the SEM images are considered because after the experimental phases when the pH was increased, the biofilm had lost its thick, confluent appearance and only isolated bacteria in single layers were visible. Nonetheless, it is possible that with longer exposure greater killing efficacy may have been achieved for both groups.

While the slow diffusion of calcium hydroxide may not necessarily result in adaptations that enhance *E. faecalis* survival compared with bacteria exposed to rapid diffusion (Section 4.5), it may result in an inadequate rise in pH during a given inter-appointment timeframe when the medication is in place. Slow diffusion may also not allow for adequate time at the maximum pH required to achieve optimal killing. In this respect, a focus on improving the diffusibility of commercial calcium hydroxide medicaments may improve their efficacy. This improvement may occur by shortening the time period it takes to reach a maximum pH and by increasing the maximum pH that can be reached in the

peripheral areas of the pulp-dentine complex during an inter-appointment time period. It has been shown that vehicles or solvents can have a significant impact on hydroxyl ion diffusion and thus new and improved solvents may be an area for further research (Fava and Saunders, 1999).

4.7 SEM Imaging

In addition to the quantitative viability counts, SEM and confocal microscopy were also conducted to qualitatively view the effect of increasing the pH on the biofilm. Together these three methods of analysis represent a comprehensive assessment of biofilms (Donlan and Costerton, 2002). SEM analysis of the control specimens revealed a confluent layer of bacterial biofilm. The biofilm appeared to be thick although it was not possible to establish how thick as no cross sectioned samples were prepared. Extra-cellular matrix material was noted. In different specimens and even in different images of the same specimen, this material appeared as either distinct fibrous connections or a more amorphous material. These differences probably represent different constituents of the extracellular matrix which can include polysaccharide, proteinaceous material or extra-cellular DNA (Branda *et al*., 2005).

SEM images of dentine disc surfaces containing biofilm after pH challenge gave variable images similar to the variable results of viability counts. Some discs showed a consistent scattering of bacterial cells over their surfaces while others showed none or only a few isolated bacteria. Discs that did show bacteria over the surface were only one cell layer thick and often appeared to have extracellular fibrous structures attaching them to the dentine surface. Such structures are suggestive of extra-cellular DNA that has been described in the extra-cellular matrix of other bacterial biofilms (Bockelmann *et al*., 2006). Furthermore, attachment of cells to the dentine disc surfaces may indicate a biofilm initiation role of possible extra-cellular DNA for *E. faecalis* similar to that reported for *Pseudomonas aueriginosa* (Whitchurch *et al*., 2002). Identification of such biofilm components, especially if involved in initiation and attachment, may make good targets for future anti-microbial agents and warrants further research.

It is not possible to tell from SEM imaging whether the cells attached to the disc surfaces after pH challenge were viable or not. However, as they appeared to be attached to the dentine, they may represent the interface layer of the original biofilm seen in the control specimens. Given the physical and physiological protections conferred by the biofilm state, these bacteria may be the survivors seen with viability testing following the experimental phases of the study.

4.8 Confocal Laser Microscopy

Confocal microscopy was carried out using a commercial staining product LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, CA, USA), which stains live bacteria green and dead bacteria red. The product contains two stains, SYTO®9 and propidium iodide which bind to and stain nucleic acids and then fluoresce when excited by laser light at certain wavelengths. However, propidium iodide (red stain), has a higher affinity for nucleic acids and thus is able to displace SYTO®9 (green stain) when it gains access to the cytoplasm. While the green stain is able to pass through an intact cell membrane the propidium iodide is not able to unless the membrane becomes porous. Thus, staining by the propidium iodide indicates a non-viable cell. The modified protocol used for staining the biofilm was specific for this study but based on manufacturer's guidelines.

Comparing the results of the confocal microscopy was interesting. As expected, the control discs grown at pH 8.1 in unbuffered THB revealed a dense biofilm with a predominance of distinctly green staining, viable bacteria and only small numbers of red, non-viable bacteria. This appearance was consistent over the whole disc surfaces. In contrast, when the pH was increased to 11.5 rapidly there appeared a greater predominance of dead bacteria. However, in this group the appearance of some indeterminate cells also occurred. These cells appeared more orange or yellow in appearance and some appeared to contain both green and red stain. Unfortunately, this was unable to be compared with the pH 11.5 slow group due to a microscope malfunction on the day the flow cell was processed. Despite the appearance of a few cells in the rapid increase to pH 11.5 group that were difficult to classify, confocal microscopy with *Bac*light initially

looked promising for delineating viable from non-viable bacteria. However, when the maximum pH was increased to 12.5 the staining became more challenging to interpret. Determining whether a cell was red or green was very difficult. While some appeared distinctly red or distinctly green and were probably dead and live cells respectively, a lot appeared yellow and on further magnification most cells appeared to have taken up both Syto 9 and propidium iodide. Not only were the cells staining but the dentine surfaces were also binding SYTO®9 stain resulting in a background with a non-specific green fluorescence.

The most obvious observation seen when comparing the confocal images of the control and pH 12.5 rapid and slow increase samples was the reduction in density of the biofilm. It is reasonable to speculate this difference is a result of gross cell death demonstrated with viability plating. However, if most bacteria have been lost from the sample biofilms, what is the state of residual bacteria seen under confocal microscopy? Unfortunately, as mentioned, overall LIVE/DEAD *Bac*light staining seems inconclusive given most cells appeared either yellow or had both the appearance of red and green stains within their cytoplasm. Clearly, the presence of some red, propidium iodide stain in most cells suggests a leaky cell membrane. However, given the higher affinity of propidium iodide for nucleic acids, it would have been expected that leaky cells would stain completely red. It is interesting to speculate whether this staining phenomenon represents an only limited porosity and thus an intermediate state between a viable and non-viable cell ie. an "unhealthy" cell but still possibly a viable one. The results of viability testing do not support this speculation. The results of viability counts were generally less than the number of cells seen on disc surfaces with confocal microscopy. This would suggest that most of the ambiguously staining cells are probably dead. An alternative but controversial explanation could be that the residual cells are actually viable but in a non-culturable state. However, this is a concept that has not been yet been conclusively proven and was not investigated in this study. Future studies might utilize Reverse Transcriptase PCR to monitor transcription in cells of questionable viability.

Limitations of *Bac*light staining have been reported. Under stress conditions Boulos *et al.* (1999) showed that different colour fluorescence occurred including yellow and orange. This was taken to represent varying degrees of cell membrane porosity and thus variation in the amount of propidium iodide able to enter cells. Berney *et al.* (2007) have also described intermediate states of staining where cells of some bacteria, when under stress, are dominated by neither SYTO®9 or propidium iodide stains. Both stains were shown to be present and of similar fluorescence intensity. However, they used an ultra-violet radiation stress rather than the high pH stress used in the present study. The *Bac*light system uses loss of cell integrity as the surrogate for loss of viability. However, this may not always be the case and cells that have suffered membrane damage may be capable of repair (Tse, 2009). Knowledge of what degree of membrane damage is required prior to loss of viability will help with the interpretation of these intermediate states. Once again, use of Reverse Transcriptase PCR might be utilized to determine viability in such situations. In this study hydroxyl ions may also have damaged the binding sites of stains on nucleic acids and thus influenced subsequent fluorescence (Tse, 2009). The rinsing protocol used (Section 2.8) would have reduced the pH of harvested biofilm suspensions but the cytoplasmic membrane damage may have been done during the experimental phases.

4.9 Limitations of Study

While many attempts have been made in the present study to mimic the *in vivo* situation, the extrapolation of the results to the *in vivo* situation must be made with caution. A number of factors are indeed likely to be quite different in this study to that found *in vivo.*

Calcium hydroxide is the primary medicament used in endodontics today. Its effect is mediated by its high pH while the calcium ion is not considered to have any antimicrobial impact. However, calcium hydroxide was not used in this study because it would not have been possible to precisely control the pH rise necessary for our experimental protocol due to its poor solubility. Hydroxyl ions would have been rapidly used up by reacting with the biofilm and subsequently

its alkaline pH would decrease. Clinically, this is not a problem because calcium hydroxide is used in a paste form which has an undissolved reservoir of solute present that would constantly replenish the depleted hydroxyl concentration. However, a paste cannot be easily pumped through a flow cell and thus buffered alkaline solutions were necessary. Buffered media could be prepared precisely to give the pH readings required. This allowed a controlled increase in flow cell pH which was important to achieve the aims of this study. Furthermore, the use of buffered growth medium ensured a stable pH and an ability to maintain the desired pH within a reasonable range despite the production of acidic end products as a result of bacterial metabolism. The typical growth curves achieved with this buffered growth medium over the range of pH used (Section 3.3) suggested that the glycine-NaCl and NaOH buffer did not have an inhibitory effect on the growth of *E. faecalis*.

The flow cell biofilm model has been used in previous studies. It is considered that the shear forces created by a constant flow of nutrient through the flow cell would create a resilient biofilm that more closely resembles biofilms found in nature (Donlan and Costerton, 2002, Dunavant *et al*., 2006). The necessity to create firm bonds to surfaces and other bacteria in order to resist the shear stresses of the constant flow is thought to produce a true biofilm. This is different to another common biofilm model that simply grows biofilms on nitro-cellulose disks laid on agar (Abdullah *et al*., 2005). While shear stresses caused by fluid movement helps create a more "realistic" biofilm, it is recognized that a mono-species biofilm was used which qualitatively may or may not represent growth *in vivo*. Only limited qualitative analysis of naturally occurring endodontic biofilms has been carried out. (Nair *et al*., 2005, Nair, 1987, Ricucci and Siqueira, 2010). However, the decision to use a mono-species *E. faecalis* biofilm was considered clinically relevant based on its frequent isolation in persistent infections of root filled teeth (Molander *et al*., 1998, Sundqvist *et al*., 1998). While primary endodontic infections are generally considered multi-species infections, culture studies have generally found that failed endodontic cases have considerably fewer species present per case and the presence of only 1-2 species per canal was not an uncommon finding. Indeed, Sundqvist *et al.* (1998) found that when

E. faecalis was isolated from failed root canal treated teeth, which was in 38% of cases, it was always the sole isolate.

Recommendations for the clinical timeframe of intracanal medication vary. As already discussed in Section 4.2, the one week duration of the experimental phase of this study was based on an *in vivo* study by Sjögren *et al.* (1991). However, clinical recommendations from 1-4 weeks have been made (Byström *et al*., 1985, Shuping *et al*., 2000, Sjögren *et al*., 1991). Longer durations of the experimental phase may have resulted in a greater incidence of discs with 100% elimination of attached bacteria than what was achieved. pH 11.5 may also have become as effective as pH 12.5 over a longer exposure time. Whether the duration of pH exposure enhances chances of adaptation and survival by *E. faecalis* to high pH is a possible direction of future research.

4.10 Directions for Future Research

While this study has fulfilled its aims, as with most research, it has also raised a number of questions towards which future research may be directed.

The few bacteria that did survive exposure to high pH media within the flow cells have been kept frozen for future experimentation. These bacteria may, through their survival, represent a population that may have developed a phenotypic adaptation to better cope with alkaline stress irrespective of the speed at which that pH rose. If this were the case, these bacteria would exhibit better survival to increases in pH than the original test organism if the same experiments were repeated. Alternatively, they may represent a persister population as described by Lewis (2007) (Section 4.4). To fulfill the definition of persister cells these bacteria would have the same susceptibility to increases in pH as the original test organism if the same experiment was repeated. Repeating the same flow cell biofilm growth experiments comparing the original test organism with the surviving bacteria from this study may shed light on whether potential phenotypic survival adaptations are permanent or transient.

Clinically, the conditions within the root canal prior to calcium hydroxide medication will vary from canal to canal. Differences might include the types of

bacteria present, anatomical variations between canals and the extent and quality of initial instrumentation and irrigation. Such factors may alter the microbial load, nutritional access and antimicrobial effectiveness. In this experiment we tried to control most variables in order to focus on the effect of pH. This included control of nutrition by including Todd Hewitt Broth in our buffer solution. In reality, it cannot be known how much nutrition is available within the root canal and this probably varies from case to case. Follow-up experiments to this study might include the effect of other variables such as nutrition. In the presence of nutrition, more energy may allow the bacteria to adapt better to the pH stress. Without such energy bacteria may be more susceptible to increases in pH stress. On the other hand, starved bacteria have been demonstrated to be more resistant to some antimicrobial challenges and thus starvation may actually increase survival (Portenier *et al.*, 2005). Possible adaptive stress responses are probably multi-factorial and thus comparing different variables or combinations of variables is necessary to get a clearer picture of bacterial responses to endodontic treatment.

Finally, confocal microscopy and the use of the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, CA, USA), also raised some interesting questions in this study. The appearance of bacteria with indeterminate staining raises the unanswered question of whether such bacteria are alive or dead. With the *Bac*Light stain, the live dead dichotomy may be overly simplistic. The propidium iodide (red) stain certainly demonstrates a loss of membrane integrity but not necessarily the extent of such damage nor whether such bacteria are capable of recovering or not. Isolating and investigating the recovery or death of damaged bacteria would be of clinical and scientific relevance when investigating residual endodontic infections post treatment.

4.11 Conclusion

A number of conclusions can be made from this work but must be considered with an appreciation of the limitations of the study. Potential extrapolation to the clinical situation may be speculated upon with caution but would require further *in vivo* research to validate the findings. It would appear from this study that *E.*

faecalis does not adapt and develop a greater resistance to high pH following a slow rise in pH compared with a rapid rise over seven days. As a result, it would not seem that the use of a high pH medicament in root canals will lead to the development of a more resistant phenotype in some *E. faecalis* bacteria over others based on their location within the root canal.

No pH protocol tested in this study resulted in complete elimination of the bacterial biofilm. However, as expected, pH 12.5 was more effective in reducing bacterial numbers compared to pH 11.5. Clinically, calcium hydroxide medicament protocols should aim to achieve the maximum pH rise *in vivo*. Such protocols might include allowing adequate time for diffusion and removing as much debris as possible prior to placement of the medicament to reduce a buffering effect.

The use of qualitative imaging enhanced the cell viability analysis. Use of these methods raises interesting questions and can provide prompts for future research into explaining the response to very high pH challenges. For example, the *Bac*light® staining protocol seems to indicate a possible incomplete breakdown in cell membrane integrity while the nature of bacterial surface and inter-cellular attachment seen in SEM images may have a role in aiding survival.

Appendix

Flow Cell Viability Counts and Protein Assays

Control

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	1.2E+07	37.3	3.2E+05
2	4.9E+05	41.6	1.2E+04
3	1.5E+06	22.9	6.5E+04
4	1.4E+07	41.0	3.5E+05
5	1.6E+06	22.7	7.0E+04
Average			1.6E+05

Flow cell 2- pH 11.5 Rapid

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	7.7E+03	21.77	3.5E+02
2	3.2E+03	12.86	2.4E+02
3	4.4E+02	7.45	5.8E+01
4	1.3E+03	12.62	1.0E+02
5	2.4E+02	8.12	3.0E+01
Average			1.6E+02

Flow cell 3- pH 11.5 Slow

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	1.4E+04	20.60	7.0E+02
2	1.2E+03	12.13	9.5E+01
3	3.2E+03	14.95	2.1E+02
4	1.2E+03	12.86	8.9E+01
5	3.4E+03	24.20	1.4E+02
6	2.4E+04	12.94	1.8E+03
Average			5.1E+02

Flow cell 4- pH 12.5 Rapid

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	1.4E+04	20.60	7.0E+02
2	1.2E+03	12.13	9.5E+01
3	3.2E+03	14.95	2.1E+02
4	1.2E+03	12.86	8.9E+01
5	3.4E+03	24.20	1.4E+02
6	2.4E+04	12.94	1.8E+03
Average			5.1E+02

Flow cell 5- pH 12.5 Slow

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	203.8	14.46	14.09
2	27.5	11.22	2.45
3	1.3	10.83	0.12
4	1.3	9.36	0.13
5	68.8	9.99	6.88
6	5.0	10.65	0.47
7	53.8	9.57	5.62
Average			4.25

Flow cell 6- pH 12.5 Slow (3 Disc Sample)

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	2.4E+03	21.20	114.4
2	1.8E+03	16.85	105.3
3	2.6E+02	12.84	19.9
Average			79.9

Flow cell 7- pH 12.5 Slow (3 Disc Sample)

Sample	Viable Count (cfu/ml)	Protein (μg/ml)	Viability (cfu /mg)
1	6.3E+02	21.53	29.0
2	5.6E+02	19.84	28.2
3	2.7E+03	33.17	79.9

Bibliography

AAS, J. A., PASTER, B. J., STOKES, L. N., OLSEN, I. & DEWHIRST, F. E. (2005) Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology,* 43**,** 5721-5732.

ABBOTT, P. V. (1990) Medicaments: aids to success in endodontics. Part 2. Clinical recommendations. *Australian Dental Journal,* 35**,** 491-496.

ABBOTT, P. V., HUME, W. R. & HEITHERSAY, G. S. (1989) Barriers to diffusion of Ledermix paste in radicular dentine. *Endod Dent Traumatol,* 5**,** 98-104.

ABDULLAH, M., NG, Y. L., GULABIVALA, K., MOLES, D. R. & SPRATT, D. A. (2005) Susceptibilties of two Enterococcus faecalis phenotypes to root canal medications. *Journal of Endodontics,* 31**,** 30-36.

AKPATA, E. & BLECHMAN, H. (1982) Bacterial invasion of pulpal dentin wall *in vitro*. *Journal of Dental Research,* 61**,** 435-438.

ANDERL, J., FRANKLIN, M. & STEWART, P. (2000) Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy,* 44**,** 1818-1824.

APPELBE, O. & SEDGLEY, C. (2007) Effects of prolonged exposure to alkaline pH on Enterococcus faecalis survival and specific gene transcripts. *Oral Microbiology and Immunology,* 22**,** 169-174.

BAUMGARTNER, J. & FALKLER JR, W. (1991) Bacteria in the apical 5 mm of infected root canals. *Journal of Endodontics,* 17**,** 380-383.

BEHNEN, M. J., WEST, L. A., LIEWEHR, F. R., BUXTON, T. B. & MCPHERSON, J. C., 3RD (2001) Antimicrobial activity of several calcium hydroxide preparations in root canal dentin. *Journal of Endodontics,* 27**,** 765-7.

BELTZ, R., TORABINEJAD, M. & POURESMAIL, M. (2003) Quantitative analysis of the solubilizing action of MTAD, sodium hypochlorite, and EDTA on bovine pulp and dentin. *Journal of Endodontics,* 29**,** 334-337.

BERNEY, M., HAMMES, F., BOSSHARD, F., WEILENMANN, H. U. & EGLI, T. (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied and Environmental Microbiology,* 73**,** 3283-3290.

BOCKELMANN, U., JANKE, A., KUHN, R., NEU, T. R., WECKE, J., LAWRENCE, J. R. & SZEWZYK, U. (2006) Bacterial extracellular DNA forming a defined network like structure. *FEMS Microbiology Letters,* 262**,** 31-38.

BOOTH, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiology and Molecular Biology Reviews,* 49**,** 359-378.

BOULOS, L., PREVOST, M., BARBEAU, B., COALLIER, J. & DESJARDINS, R. (1999) LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods,* 37**,** 77-86.

BRANDA, S. S., VIK, A., FRIEDMAN, L. & KOLTER, R. (2005) Biofilms: the matrix revisited. *TRENDS in Microbiology,* 13**,** 20-26.

BRANDLE, N., ZEHNDER, M., WEIGER, R. & WALTIMO, T. (2008) Impact of growth conditions on susceptibility of five microbial species to alkaline stress. *Journal of Endodontics,* 34**,** 579-582.

BYSTRÖM, A., CLAESSON, R. & SUNDQVIST, G. (1985) The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Dental Traumatology,* 1**,** 170-175.

BYSTRÖM, A., HAPPONEN, R., SJÖGREN, U. & SUNDQVIST, G. (1987) Healing of periapical lesions of pulpless teeth after endodontic treatment with controlled asepsis. *Dental Traumatology,* 3**,** 58-63.

BYSTRÖM, A. & SUNDQVIST, G. (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *European Journal of Oral Sciences,* 89**,** 321-328.

BYSTRÖM, A. & SUNDQVIST, G. (1983) Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surgery, Oral Medicine, Oral Pathology,* 55**,** 307-312.

CARRIGAN, P. J., MORSE, D. R., FURST, M. L. & SINAI, I. H. (1984) A scanning electron microscopic evaluation of human dentinal tubules according to age and location. *Journal of Endodontics,* 10**,** 359-363.

CHÁVEZ DE PAZ, L. (2007) Redefining the persistent infection in root canals: possible role of biofilm communities. *Journal of Endodontics,* 33**,** 652-662.

CHÁVEZ DE PAZ, L., DAHLÈN, G., MOLANDER, A., MÖLLER, A. & BERGENHOLTZ, G. (2003) Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. *International Endodontic Journal,* 36**,** 500-508.

CHÁVEZ DE PAZ, L. E., BERGENHOLTZ, G., DAHLÈN, G. & SVENSÅTER, G. (2007) Response to alkaline stress by root canal bacteria in biofilms. *International Endodontic Journal,* 40**,** 344-55.

CHU, F., LEUNG, W., TSANG, P., CHOW, T. & SAMARANAYAKE, L. (2006) Identification of cultivable microorganisms from root canals with apical periodontitis following two-visit endodontic treatment with antibiotics/steroid or calcium hydroxide dressings. *Journal of Endodontics,* 32**,** 17-23.

CHU, F., TSANG, C., CHOW, T. & SAMARANAYAKE, L. (2005) Identification of cultivable microorganisms from primary endodontic infections with exposed and unexposed pulp space. *Journal of Endodontics,* 31**,** 424-429.

CIARDI, J., BEAMAN, A. & WITTENBERGER, C. (1977) Purification, resolution, and interaction of the glucosyltransferases of Streptococcus mutans 6715. *Infection and Immunity,* 18**,** 237-246.

COSTERTON, J., GEESEY, G. & CHENG, K. (1978) How bacteria stick. *Scientific American,* 238**,** 86-95.

COSTERTON, J., LEWANDOWSKI, Z., CALDWELL, D., KORBER, D. & LAPPIN-SCOTT, H. (1995) Microbial biofilms. *Annual Reviews in Microbiology,* 49**,** 711-745.

COSTERTON, J., STEWART, P. & GREENBERG, E. (1999) Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-1322.

CVITKOVITCH, D., LI, Y. & ELLEN, R. (2003) Quorum sensing and biofilm formation in streptococcal infections. *Journal of Clinical Investigation,* 112**,** 1626-1632.

DALTON, B. C., ØRSTAVIK, D., PHILLIPS, C., PETTIETTE, M. & TROPE, M. (1998) Bacterial reduction with nickel-titanium rotary instrumentation. *Journal of Endodontics,* 24**,** 763-767.

DAS, J., BHAKOO, M., JONES, M. & GILBERT, P. (1998) Changes in the biocide susceptibility of Staphylococcus epidermidis and Escherichia coli cells associated with rapid attachment to plastic surfaces. *Journal of Applied Microbiology,* 84**,** 852-858.

DE BEER, D., STOODLEY, P., ROE, F. & LEWANDOWSKI, Z. (1994) Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and Bioengineering,* 43**,** 1131-1138.

DISTEL, J., HATTON, J. & GILLESPIE, M. (2002) Biofilm formation in medicated root canals. *Journal of endodontics,* 28**,** 689-693.

DONLAN, R. & COSTERTON, J. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews,* 15**,** 167-193.

DUNAVANT, T., REGAN, J., GLICKMAN, G., SOLOMON, E. & HONEYMAN, A. (2006) Comparative evaluation of endodontic irrigants against Enterococcus faecalis biofilms. *Journal of endodontics,* 32**,** 527-531.

ENGSTRÖM, B. & FROSTELL, G. (1964) Correlation of positive cultures with the prognosis for root canal treatment. *Odontologisk Revy*, 15, 239-270.

EVANS, M., DAVIES, J., SUNDQVIST, G. & FIGDOR, D. (2002) Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. *International Endodontic Journal,* 35**,** 221-228.

FABRICIUS, L., DAHLÈN, G., HOLM, S. & MÖLLER, A. (1982a) Influence of combinations of oral bacteria on periapical tissues of monkeys. *European Journal of Oral Sciences,* 90**,** 200-206.

FABRICIUS, L., DAHLÈN, G. & MÖLLER, A. (1982b) Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *European Journal of Oral Sciences,* 90**,** 134-144.

FAVA, L. R. & SAUNDERS, W. P. (1999) Calcium hydroxide pastes: classification and clinical indications. *International Endodontic Journal,* 32**,** 257-282.

FIGDOR, D., DAVIES, J. & SUNDQVIST, G. (2003) Starvation survival, growth and recovery of Enterococcus faecalis in human serum. *Oral Microbiology and Immunology,* 18**,** 234-239.

FLAHAUT, S., HARTKE, A., GIARD, J. & AUFFRAY, Y. (1997) Alkaline stress response in Enterococcus faecalis: adaptation, cross-protection, and changes in protein synthesis. *Applied and Environmental Microbiology,* 63**,** 812-814.

FOGEL, H. M., MARSHALL, F. J. & PASHLEY, D. H. (1988) Effects of distance from the pulp and thickness on the hydraulic conductance of human radicular dentin. *Journal of Dental Research,* 67**,** 1381-1385.

FOUAD, A., BARRY, J., CAIMANO, M., CLAWSON, M., ZHU, Q., CARVER, R., HAZLETT, K. & RADOLF, J. (2002) PCR-based identification of bacteria associated with endodontic infections. *Journal of Clinical Microbiology,* 40**,** 3223-3231.

FRANZ, C. M. A. P., STILES, M. E., SCHLEIFER, K. H. & HOLZAPFEL, W. H. (2003) Enterococci in foods--a conundrum for food safety. *International Journal of Food Microbiology,* 88**,** 105-122.

FUX, C., COSTERTON, J., STEWART, P. & STOODLEY, P. (2005) Survival strategies of infectious biofilms. *TRENDS in Microbiology,* 13**,** 34-40.

GALVAN, D. A., CIARLONE, A. E., PASHLEY, D. H., KULILD, J. C., PRIMACK, P. D. & SIMPSON, M. D. (1994) Effect of smear layer removal on the diffusion permeability of human roots. *Journal of Endodontics,* 20**,** 83-86.

GARBEROGLIO, R. & BRÄNNSTRÖM, M. (1976) Scanning electron microscopic investigation of human dentinal tubules. *Archives of Oral Biology,* 21**,** 355-62.

GIRAFFA, G. (2002) Enterococci from foods. *FEMS Microbiology Reviews,* 26**,** 163-171.

GOLD, O., JORDAN, H. & VAN HOUTE, J. (1975) The prevalence of enterococci in the human mouth and their pathogenicity in animal models. *Archives of Oral Biology,* 20**,** 473-477.

GOMES, B., FERRAZ, C., ME, V., BERBER, V., TEIXEIRA, F. & SOUZA FILHO, F. (2001) *In vitro* antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. *International Endodontic Journal,* 34**,** 424-428.

GOMES, B., PINHEIRO, E., GADE-NETO, C., SOUSA, E., FERRAZ, C., ZAIA, A., TEIXEIRA, F. & SOUZA-FILHO, F. (2004) Microbiological examination of infected dental root canals. *Oral microbiology and Immunology,* 19**,** 71-76.

GOMES, B., SOUZA, S., FERRAZ, C., TEIXEIRA, F., ZAIA, A., VALDRIGHI, L. & SOUZA-FILHO, F. (2003) Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine root dentine *in vitro*. *International Endodontic Journal,* 36**,** 267-275.

GORDON, T., DAMATO, D. & CHRISTNER, P. (1981) Solvent effect of various dilutions of sodium hypochlorite on vital and necrotic tissue. *Journal of Endodontics,* 7**,** 466-469.

HAAPASALO, H. K., SIREN, E. K., WALTIMO, T. M., ØRSTAVIK, D. & HAAPASALO, M. P. (2000) Inactivation of local root canal medicaments by dentine: an *in vitro* study. *International Endodontic Journal,* 33**,** 126-31.

HAAPASALO, M. & ØRSTAVIK, D. (1987) *In vitro* infection and disinfection of dentinal tubules. *J Dent Res,* 66**,** 1375-9.

HAAPASALO, M., QIAN, W., PORTENIER, I. & WALTIMO, T. (2007) Effects of dentin on the antimicrobial properties of endodontic medicaments. *Journal of Endodontics,* 33**,** 917-25.

HALL-STOODLEY, L., COSTERTON, J. & STOODLEY, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology,* 2**,** 95-108.

HANCOCK, H. (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics,* 91**,** 579-586.

HOLDEMAN, L. & Moore, W. E. C. (1975) *Anaerobe Laboratory Manual*, 3rd ed. Virginia Polytechnic and State University, Anaerobe Laboratory, Blacksburg, Va.

HUBBLE, T., HATTON, J., NALLAPAREDDY, S., MURRAY, B. & GILLESPIE, M. (2003) Influence of Enterococcus faecalis proteases and the collagen binding protein, Ace, on adhesion to dentin. *Oral Microbiology and Immunology,* 18**,** 121-126.

IKE, Y. & CLEWELL, D. (1992) Evidence that the hemolysin/bacteriocin phenotype of Enterococcus faecalis subsp. zymogenes can be determined by plasmids in different incompatibility groups as well as by the chromosome. *Journal of Bacteriology,* 174**,** 8172-8177.

JETT, B. & GILMORE, M. (1990) The growth-inhibitory effect of the Enterococcus faecalis Bacteriocin Encoded by pADl Extends to the Oral Streptococci. *Journal of Dental Research,* 69**,** 1640-1645.

KAKEHASHI, S., STANLEY, H. & FITZGERALD, R. (1965) The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surgery, Oral Medicine, Oral Pathology,* 20**,** 340-349.

KAKINUMA, Y. (1998) Inorganic cation transport and energy transduction in Enterococcus hirae and other streptococci. *Microbiology and Molecular Biology Reviews,* 62**,** 1021-1045.

KAKINUMA, Y. & IGARASHI, K. (1995) Potassium/proton antiport system of growing Enterococcus hirae at high pH. *Journal of Bacteriology,* 177**,** 2227-2229.

KAYAOGLU, G., ERTEN, H. & ØRSTAVIK, D. (2005) Growth at high pH increases Enterococcus faecalis adhesion to collagen. *International Endodontic Journal,* 38**,** 389-396.

KAYAOGLU, G. & ØRSTAVIK, D. (2004) Virulence factors of Enterococcus faecalis: relationship to endodontic disease. *Critical Reviews in Oral Biology & Medicine,* 15**,** 308-20.

KOWALSKI, W. J., KASPER, E. L., HATTON, J. F., MURRAY, B. E., NALLAPAREDDY, S. R. & GILLESPIE, M. J. (2006) Enterococcus faecalis adhesin, Ace, mediates attachment to particulate dentin. *Journal of Endodontics,* 32**,** 634-637.

KVIST, T., MOLANDER, A., DAHLÈN, G. & REIT, C. (2004) Microbiological evaluation of one-and two-visit endodontic treatment of teeth with apical periodontitis: a randomized, clinical trial. *Journal of Endodontics,* 30**,** 572-576.

LAW, A. & MESSER, H. (2004) An evidence-based analysis of the antibacterial effectiveness of intracanal medicaments. *Journal of Endodontics,* 30**,** 689-694.

LEONARDO, M., ROSSI, M., SILVA, L., ITO, I. & BONIF-CIO, K. (2002) EM evaluation of bacterial biofilm and microorganisms on the apical external root surface of human teeth. *Journal of Endodontics,* 28**,** 815-818.

LEWIS, K. (2007) Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology,* 5**,** 48-56.

LLEO, M., PIEROBON, S., TAFI, M. C., SIGNORETTO, C. & CANEPARI, P. (2000) mRNA detection by reverse transcription-PCR for monitoring viability over time in an Enterococcus faecalis viable but nonculturable population maintained in a laboratory microcosm. *Applied and environmental microbiology,* 66**,** 4564.

LOVE, R. M. (1996) Regional variation in root dentinal tubule infection by Streptococcus gordonii. *Journal of Endodontics,* 22**,** 290-293.

LOVE, R. M. (2001) Enterococcus faecalis- a mechanism for its role in endodontic failure. *International Endodontic Journal,* 34**,** 399-405.

LYNCH, J. L., RAPHAEL, S. S., MELLOR, L. D., SPARE, P. D., INWOOD, M. J. H. (1969) *Medical laboratory technology and Clinical pathology*, 2nd ed. Saunders, Ontario. Appendix F, 1300.

MARSH, P. D., BOWDEN, G. H. W (2000) Microbial community interactions in biofilms; in Allison DG, Gilbert P, Lappin-Scott HM, Wilson M (eds): Community Structure and Co-Operation in Biofilms. Society for

Microbiology Symposium 59. Cambridge University Press, Cambridge, 167–198.
McGURKIN-SMITH, R., TROPE, M., CAPLAN, D. & SIGURDSSON, A. (2005) Reduction of intracanal bacteria using GT rotary instrumentation, 5.25% NaOCl, EDTA, and Ca (OH) 2. *Journal of Endodontics,* 31**,** 359-363.
McHUGH, C. P., ZHANG, P., MICHALEK, S. & ELEAZER, P. D. (2004) pH required to kill Enterococcus faecalis *in vitro*. *Journal of Endodontics,* 30**,** 218-219.
MOLANDER, A., REIT, C., DAHLÈN, G. & KVIST, T. (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal,* 31**,** 1-7.
MOLANDER, A., WARFVINGE, J., REIT, C. & KVIST, T. (2007) Clinical and radiographic evaluation of one-and two-visit endodontic treatment of asymptomatic necrotic teeth with apical periodontitis: a randomized clinical trial. *Journal of Endodontics,* 33**,** 1145-1148.
MÖLLER, A. (1966) Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. *Odontologisk Tidskrift,* 74, 1-380.
MÖLLER, A., FABRICIUS, L. & DAHLÈN, N., G (1981) Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *European Journal of Oral Sciences,* 89**,** 475-484.
MOLVEN, O., OLSEN, I. & KEREKES, K. (1991) Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. *Dental Traumatology,* 7**,** 226-229.
NAIR, P. (2004) Pathogenesis of apical periodontitis and the causes of endodontic failures. *Critical Reviews in Oral Biology & Medicine,* 15**,** 348.
NAIR, P., HENRY, S., CANO, V. & VERA, J. (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one visit" endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology,* 99**,** 231-252.
NAIR, P., SJÖGREN, U., KREY, G., KAHNBERG, K. & SUNDQVIST, G. (1990) Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *Journal of Endodontics,* 16**,** 580-588.
NAIR, R. (1987) Light and electron microscopic studies of root canal flora and periapical lesions. *Journal of Endodontics,* 13**,** 29-39.
NALLAPAREDDY, S., QIN, X., WEINSTOCK, G., HOOK, M. & MURRAY, B. (2000) Enterococcus faecalis adhesin, Ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infection and Immunity,* 68**,** 5218-5224.
NERWICH, A., FIGDOR, D. & MESSER, H. H. (1993) pH changes in root dentin over a 4-week period following root canal dressing with calcium hydroxide. *J Endod,* 19**,** 302-306.
NG, Y. L., MANN, V., RAHBARAN, S., LEWSEY, J. & GULABIVALA, K. (2008) Outcome of primary root canal treatment: systematic review of the literature: Part 2. Influence of clinical factors. *International Endodontic Journal,* 41**,** 6-31.
ØRSTAVIK, D. & HAAPASALO, M. (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod Dent Traumatol,* 6**,** 142-9.

PASHLEY, D. H. (1985) Dentin-predentin complex and its permeability: physiologic overview. *Journal of Dental Research,* 64 Spec Iss No**,** 613-620.
PECIULIENE, V., REYNAUD, A., BALCIUNIENE, I. & HAAPASALO, M. (2001) Isolation of yeasts and enteric bacteria in root filled teeth with chronic apical periodontitis. *International Endodontic Journal,* 34**,** 429-434.
PETERS, L. & WESSELINK, P. (2002) Periapical healing of endodontically treated teeth in one and two visits obturated in the presence or absence of detectable microorganisms. *International Endodontic Journal,* 35**,** 660-667.
PETERS, L., WESSELINK, P., BUIJS, J. & VAN WINKELHOFF, A. (2001a) Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *Journal of Endodontics,* 27**,** 76-81.
PETERS, L. B., VAN WINKELHOFF, A. J., BUIJS, J. F. & WESSELINK, P. R. (2002) Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. *International Endodontic Journal,* 35**,** 13-21.
PETERS, O., SCHÔNENBERGER, K. & LAIB, A. (2001b) Effects of four NiTi preparation techniques on root canal geometry assessed by micro computed tomography. *International Endodontic Journal,* 34**,** 221-230.
PLUTZER, B. (2009) Efficacy of Endodontic Medicaments against an Enterococcus Faecalis biofilm. *DClinDent Thesis.* School of Dentistry, University of Adelaide, Adelaide.
PORTENIER, I., HAAPASALO, H., RYE, A., WALTIMO, T., ØRSTAVIK, D. & HAAPASALO, M. (2001) Inactivation of root canal medicaments by dentine, hydroxylapatite and bovine serum albumin. *International Endodontic Journal,* 34**,** 184-188.
PORTENIER, I., WALTIMO, T. & HAAPASALO, M. (2003) Enterococcus faecalis: the root canal survivor and star in post treatment disease. *Endodontic Topics,* 6**,** 135-159.
PORTENIER, I., WALTIMO, T., ØRSTAVIK, D. & HAAPASALO, M. (2005) The susceptibility of starved, stationary phase, and growing cells of Enterococcus faecalis to endodontic medicaments. *Journal of Endodontics,* 31**,** 380-386.
RAKITA, R., VANEK, N., JACQUES-PALAZ, K., MEE, M., MARISCALCO, M., DUNNY, G., SNUGGS, M., VAN WINKLE, W. & SIMON, S. (1999) Enterococcus faecalis bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infection and Immunity,* 67**,** 6067-6075.
RAZAVI, A., GM¸R, R., IMFELD, T. & ZEHNDER, M. (2007) Recovery of Enterococcus faecalis from cheese in the oral cavity of healthy subjects. *Oral Microbiology and Immunology,* 22**,** 248-251.
RICUCCI, D. & SIQUEIRA, J. (2010) Biofilms and Apical Periodontitis: Study of Prevalence and Association with Clinical and Histopathologic Findings. *Journal of Endodontics,* 36**,** 1277-1288.
RÔÇAS, I. & SIQUEIRA, J. (2004) Association of Enterococcus faecalis with different forms of periradicular diseases. *Journal of Endodontics,* 30**,** 315-320.

RÔÇAS, I., SIQUEIRA JR, J., ANDRADE, A. & UZEDA, M. (2003) Oral treponemes in primary root canal infections as detected by nested PCR. *International Endodontic Journal,* 36**,** 20-26.

ROZDZINSKI E., MARRE R., SUSA M., WIRTH R., MUSCHOLL-SILBERHORN A. (2001). Aggregation substance mediated adherence of Enterococcus faecalis to immobilized extracellular matrix pro- teins. *Microbial Pathogenesis* 30,211-220.

SAFAVI, K. & NAKAYAMA, T. A. (2000) Influence of mixing vehicle on dissociation of calcium hydroxide in solution. *J Endod,* 26**,** 649-651.

SAFAVI, K. E., SPÅNGBERG, L. S. & LANGELAND, K. (1990) Root canal dentinal tubule disinfection. *J Endod,* 16**,** 207-210.

SAKAMOTO, M., RÙÁAS, I., SIQUEIRA JR, J. & BENNO, Y. (2006) Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. *Oral microbiology and Immunology,* 21**,** 112-122.

SAKAMOTO, M., SIQUEIRA JR, J., RÙÁAS, I. & BENNO, Y. (2008) Molecular analysis of the root canal microbiota associated with endodontic treatment failures. *Oral microbiology and Immunology,* 23**,** 275-281.

SASSONE, L., FIDEL, R., FAVERI, M., GUERRA, R., FIGUEIREDO, L., FIDEL, S. & FERES, M. (2008) A microbiological profile of symptomatic teeth with primary endodontic infections. *Journal of Endodontics,* 34**,** 541-545.

SATHORN, C., PARASHOS, P. & MESSER, H. (2005) Effectiveness of single versus multiple visit endodontic treatment of teeth with apical periodontitis: a systematic review and meta analysis. *International Endodontic Journal,* 38**,** 347-355.

SATHORN, C., PARASHOS, P. & MESSER, H. H. (2007a) How Useful Is Root Canal Culturing in Predicting Treatment Outcome? *Journal of Endodontics,* 33**,** 220-225.

SATHORN, C., PARASHOS, P. & MESSER, H. (2007b) Antibacterial efficacy of calcium hydroxide intracanal dressing: a systematic review and meta-analysis. *International Endodontic Journal,* 40**,** 2-10.

SAUER, K., CAMPER, A., EHRLICH, G., COSTERTON, J. & DAVIES, D. (2002) Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology,* 184**,** 1140-1154.

SCHAFER, E. & BOSSMANN, K. (2005) Antimicrobial efficacy of chlorhexidine and two calcium hydroxide formulations against Enterococcus faecalis. *Journal of Endodontics,* 31**,** 53-56.

SEDGLEY, C. M., BUCK, G. & APPELBE, O. (2006a) Prevalence of Enterococcus faecalis at multiple oral sites in endodontic patients using culture and PCR. *Journal of Endodontics,* 32**,** 104-109.

SEDGLEY, C. M., LENNAN, S. & CLEWELL, D. (2004) Prevalence, phenotype and genotype of oral enterococci. *Oral Microbiology and Immunology,* 19**,** 95-101.

SEDGLEY, C. M., NAGEL, A., SHELBURNE, C., CLEWELL, D., APPELBE, O. & MOLANDER, A. (2005a) Quantitative real-time PCR detection of oral Enterococcus faecalis in humans. *Archives of Oral Biology,* 50**,** 575-583.

SEDGLEY, C. M., MOLANDER, A., FLANNAGAN, S., NAGEL, A., APPELBE, O., CLEWELL, D. & DAHLÈN, G. (2005b) Virulence, phenotype and genotype characteristics of endodontic Enterococcus spp. *Oral Microbiology and Immunology,* 20**,** 10-19.

SEDGLEY, C. M., NAGEL, A. C., HALL, D. & APPLEGATE, B. (2005c) Influence of irrigant needle depth in removing bioluminescent bacteria inoculated into instrumented root canals using real-time imaging *in vitro*. *International Endodontic Journal,* 38**,** 97-104

SEDGLEY, C. M., LENNAN, S. & APPELBE, O. (2005d) Survival of Enterococcus faecalis in root canals ex vivo. *International Endodontic Journal,* 38**,** 735-742.

SEDGLEY, C. M., LEE, E. H., MARTIN, M. J. & FLANNAGAN, S. E. (2008) Antibiotic resistance gene transfer between Streptococcus gordonii and Enterococcus faecalis in root canals of teeth ex vivo. *Journal of Endodontics,* 34**,** 570-574.

SHANKAR, V., BAGHDAYAN, A., HUYCKE, M., LINDAHL, G. & GILMORE, M. (1999) Infection-derived Enterococcus faecalis strains are enriched in esp, a gene encoding a novel surface protein. *Infection and Immunity,* 67**,** 193-200.

SHUPING, G. B., ØRSTAVIK, D., SIGURDSSON, A. & TROPE, M. (2000) Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. *J Endod,* 26**,** 751-7555.

SIGNORETTO, C., DEL MAR LLEO, M., TAFI, M. & CANEPARI, P. (2000) Cell wall chemical composition of Enterococcus faecalis in the viable but nonculturable state. *Applied and Environmental Microbiology,* 66**,** 1953-1959.

SIQUEIRA, J. (2002) Patterns of microbial colonization in primary root canal infections. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics,* 93**,** 174-178.

SIQUEIRA, J. (2003) PCR methodology as a valuable tool for identification of endodontic pathogens. *Journal of dentistry,* 31**,** 333-339.

SIQUEIRA, J. (2005) Exploiting molecular methods to explore endodontic infections: Part 2- Redefining the endodontic microbiota. *Journal of Endodontics,* 31**,** 488-498.

SIQUEIRA, J. & RÔÇAS, I. (2004) Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics,* 97**,** 85-94.

SIQUEIRA, J. & RÔÇAS, I. (2010) Microbiology and Treatemnt of Endodontic Infections. In Hargreaves, K. & Cohen, S. (Eds.) *Pathways of the Pulp,* 10th Ed. Mosby, St Louis, 559-601

SIQUEIRA JR, J. & DE UZEDA, M. (1996) Disinfection by calcium hydroxide pastes of dentinal tubules infected with two obligate and one facultative anaerobic bacteria. *Journal of Endodontics,* 22**,** 674-676.

SIQUEIRA JR, J. & LOPES, H. (1999) Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. *International Endodontic Journal,* 32**,** 361-369.

SIQUEIRA JR, J., R AS, I., SOUTO, R., DE UZEDA, M. & COLOMBO, A. (2002) Actinomyces species, streptococci, and Enterococcus faecalis in primary root canal infections. *Journal of Endodontics,* 28**,** 168-172.

SIREN, E., HAAPASALO, M., RANTA, K., SALMI, P. & KEROSUO, E. (1997) Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *International Endodontic Journal,* 30**,** 91-95.

SJÖGREN, U., FIGDOR, D., PERSSON, S. & SUNDQVIST, G. (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *International Endodontic Journal,* 30**,** 297-306.

SJÖGREN, U., FIGDOR, D., SPÅNGBERG, L. & SUNDQVIST, G. (1991) The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. *International Endodontic Journal,* 24**,** 119-125.

SJÖGREN, U., HOGGLUND, B., SUNDQVIST, G. & WING, K. (1990) Factors affecting the long-term results of endodontic treatment. *Journal of Endodontics,* 16**,** 498-504.

STEWART, P. (1996) Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy,* 40**,** 2517-2522.

STEWART, P. & COSTERTON, J. (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet,* 358**,** 135-138.

SUNDQVIST, G. (1976) Bacteriological studies of necrotic dental pulps. *Thesis.* Department of Oral Microbiology, University of Umeå, Umeå

SUNDQVIST, G. (1992) Ecology of the root canal flora. *Journal of Endodontics,* 18, 427-430.

SUNDQVIST, G. & FIGDOR, D. (2003) Life as an endodontic pathogen. *Endodontic Topics,* 6**,** 3-28.

SUNDQVIST, G., FIGDOR, D., PERSSON, S. & SJÖGREN, U. (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology,* 85**,** 86-93.

SVENSÅTER, G. & BERGENHOLTZ, G. (2004) Biofilms in endodontic infections. *Endodontic Topics,* 9**,** 27-36.

TAO, L., ANDERSON, R. W. & PASHLEY, D. H. (1991) Effect of endodontic procedures on root dentin permeability. *Journal of Endodontics,* 17**,** 583-588.

TOLEDO-ARANA, A., VALLE, J., SOLANO, C., ARRIZUBIETA, M., CUCARELLA, C., LAMATA, M., AMORENA, B., LEIVA, J., PENADES, J. & LASA, I. (2001) The enterococcal surface protein, Esp, is involved in Enterococcus faecalis biofilm formation. *Applied and Environmental Microbiology,* 67**,** 4538-4545.

TRONSTAD, L., BARNETT, F. & CERVONE, F. (1990) Periapical bacterial plaque in teeth refractory to endodontic treatment. *Dental Traumatology,* 6**,** 73-77.

TROPE, M., DELANO, E. & ØRSTAVIK, D. (1999) Endodontic treatment of teeth with apical periodontitis: single vs. multivisit treatment. *Journal of Endodontics,* 25**,** 345.

TSE, A. (2009) Development of a model to determine the efficacy of antibacterial treatment in dentinal tubules. *DClinDent Thesis*, University of Melbourne, Melbourne.

TUOMANEN, E., COZENS, R., TOSCH, W., ZAK, O. & TOMASZ, A. (1986) The rate of killing of Escherichia coli by by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. *Microbiology,* 132**,** 1297-1304.

VERTUCCI, F. (1984) Root canal anatomy of the human permanent teeth. *Oral Surgery, Oral Medicine, Oral Pathology,* 58**,** 589-599.

WALTIMO, T., TROPE, M., HAAPASALO, M. & ØRSTAVIK, D. (2005) Clinical efficacy of treatment procedures in endodontic infection control and one year follow-up of periapical healing. *Journal of Endodontics,* 31**,** 863-866.

WEIGER, R., ROSENDAHL, R. & LÖST, C. (2000) Influence of calcium hydroxide intracanal dressings on the prognosis of teeth with endodontically induced periapical lesions. *International Endodontic Journal,* 33**,** 219-226.

WHITCHURCH, C. B., TOLKER-NIELSEN, T., RAGAS, P. C. & MATTICK, J. S. (2002) Extracellular DNA required for bacterial biofilm formation. *Science,* 295**,** 1487.

WICKEN, A. & KNOX, K. (1975) Lipoteichoic acids: a new class of bacterial antigen. *Science,* 187**,** 1161-1167.

WILLIAMS, J., TROPE, M., CAPLAN, D. & SHUGARS, D. (2006) Detection and quantitation of E. faecalis by real-time PCR (qPCR), reverse transcription-PCR (RT-PCR), and cultivation during endodontic treatment. *Journal of Endodontics,* 32**,** 715-721.

WU, M., R'ORIS, A., BARKIS, D. & WESSELINK, P. (2000) Prevalence and extent of long oval canals in the apical third. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology,* 89**,** 739-743.

YOUNG, G., TURNER, S., DAVIES, J., SUNDQVIST, G. & FIGDOR, D. (2007) Bacterial DNA persists for extended periods after cell death. *Journal of Endodontics,* 33**,** 1417-1420.

ZEHNDER, M. & GUGGENHEIM, B. (2009) The mysterious appearance of enterococci in filled root canals. *International Endodontic Journal,* 42**,** 277-287.