An *in-vitro* Evaluation of the Effectiveness of Endodontic Irrigants, with and without Sonic and Laser Activation, in the Eradication of *Enterococcus faecalis* Biofilm

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

Dr Aaron Nicholas Seet
BSc (Hons) (S’pore), BDS (Adel)
Contents

Declaration ............................................................................................................................ i
Acknowledgements ........................................................................................................... ii
Abstract .............................................................................................................................. 1

Introduction ......................................................................................................................... 1
Aim ....................................................................................................................................... 1
Methodology ......................................................................................................................... 1
Results .................................................................................................................................. 2
Conclusion ............................................................................................................................. 3

Chapter 1. Literature Review ............................................................................................. 4

1.1 Introduction .................................................................................................................... 4
1.2 The microbiological basis of endodontic therapy .......................................................... 4
1.3 The single versus multiple visit endodontic therapy debate .......................................... 7
1.4 Microbial root canal sampling ...................................................................................... 12
1.5 Post treatment disease .................................................................................................. 13
  1.5.1 The prevalence of post treatment disease ............................................................... 13
  1.5.2 The causes of post treatment disease .................................................................... 14
  1.5.3 The microbiology of post-treatment disease .......................................................... 16
1.6 Enterococcus faecalis ................................................................................................... 19
  1.6.1 An introduction to Enterococcus faecalis ............................................................... 19
  1.6.2 The significance of Enterococcus faecalis in infections ........................................... 20
  1.6.3 Enterococcus faecalis and endodontic infections ..................................................... 21
  1.6.4 The virulence of Enterococcus faecalis ................................................................... 21
    1.6.4.1 Aggregation substance .................................................................................... 22
    1.6.4.2 Cytolysin ........................................................................................................ 23
    1.6.4.3 Gelatinase ....................................................................................................... 24
    1.6.4.4 Surface adhesins ............................................................................................ 24
    1.6.4.5 Extracellular superoxide production ............................................................... 25
    1.6.4.6 Hyaluronidase ............................................................................................... 25
    1.6.4.7 AS-48 ............................................................................................................. 26
  1.6.5 The survival of Enterococcus faecalis within the root canal ......................................... 27
1.7 Endodontic therapy ...................................................................................................... 33
  1.7.1 The importance of chemomechanical debridement .................................................. 33
Declaration

I, Aaron Seet, 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: _________________

Aaron Seet

Date: _________     Date: __________
Acknowledgements

Firstly, I would like to express my greatest gratitude to A/Prof Peter Cathro for his guidance and support, both on a professional and personal basis. His boundless patience and encouragement, as well as the constant reminders to keep things in perspective will never be forgotten. Thank you for seeing me through some of the darkest days.

Deepest thanks to Dr Peter Zilm, for sharing his wealth of research experience. His expertise and uncanny ability to devise ingenious solutions were invaluable in seeing the research project through. I am always indebted for his assistance so that I could see the birth of my daughter while the project was running.

I would also like to extend my thanks to Prof Geoffrey Heithersay for his insights and pearls of wisdom. He has been most generous with his wealth of knowledge and I am always grateful and privileged to have been able to partake of it. His friendship and willingness to listen will always be treasured.

Many thanks also to Dr Neville Gully for his assistance and advice with the research project. I appreciate the time taken to read and correct my thesis.

I would also like to acknowledge the assistance of all the staff at the Adelaide Microscopy Centre, especially Lyn Waterhouse, who was always willing to lend a hand.

The postgraduate program would not have been the same without the friendship of Drs Barbara Plutzer, Mark Stenhouse and Jonathan Christo. It has been an honour to be in the company of such fine friends.

Thank you, Dad, Mum and Lyn for your love and always believing in me.

San, thank you for your unconditional love, patience and sacrifice so that I can fulfil my aspirations. Thank you for our beautiful daughter, Shevonne. The completion of this degree would have been empty if not for the both of you.

Finally, I would like to acknowledge the support of Dentsply and Biolase for the loan of equipment necessary for the conduct of the research, as well as financial support from the Australian Dental Research Foundation and the Australian Society of Endodontology.
Abstract

Introduction

It is well established that the causative agent of endodontic disease is the presence and growth of bacteria (Kakehashi et al., 1965; Möller et al., 1981). Therefore, eradication of bacteria is essential to prevent or eliminate apical periodontitis. Studies have shown that elimination of bacteria prior to obturation has resulted in a more favourable outcome for endodontic therapy (Sjögren et al., 1997). When endodontic treatment fails, bacteria is often isolated from the root canals of these teeth. One of the most commonly isolated bacteria is Enterococcus faecalis (Molander et al., 1998; Sundqvist et al., 1998). As such, endodontic therapy is founded upon three principles: mechanical instrumentation; irrigation with antimicrobial agents and placement of an intracanal medicament (Haapasalo et al., 2005). However, the complex anatomy of the root canal system often prevents the penetration of irrigants and medicaments into recesses that cannot be accessed by mechanical instrumentation. The advent of sonic, ultrasonic and laser instruments has led to many investigations looking at their potential for the activation of irrigants (Lee et al., 2004; de Gregorio et al., 2009; De Moor et al., 2009). However, most of these studies have concentrated on the removal of dentinal debris and smear layer (Lee et al., 2004).

Aim

The aim of this study was to evaluate and compare the effectiveness of three modes of irrigation: syringe irrigation; sonic activation and laser activation of the irrigant in eradicating E. faecalis that had been cultivated within the root canals of extracted single rooted teeth.

Methodology

A flow cell was designed and constructed. The extracted teeth were decoronated, and prepared with rotary instruments to #40 to 1 mm beyond the apex of the tooth. This was to allow nutrient media to flow through the root canals. The flow cell was connected to a nutrient reservoir containing Todd Hewitt Broth, which was pumped into the flow cell via a peristaltic pump.
The flow cell was inoculated with *E. faecalis* (ATCC 700802) and cultivated for a period of four weeks. The flow cell was then dismantled and the teeth were assigned to 6 treatment groups:
1. syringe irrigation with saline
2. syringe irrigation with 4% sodium hypochlorite
3. sonic activation of saline (EndoActivator, Dentsply)
4. sonic activation of 4% sodium hypochlorite
5. laser activation of saline (Er,Cr:YSGG Waterlase, Biolase Technology)
6. laser activation of 4% sodium hypochlorite

Teeth were irrigated with 5 ml of either saline or 4% sodium hypochlorite for 1 minute. The 4% sodium hypochlorite solution was inactivated with 5% sodium thiosulphate. Teeth that received sonic activation were irrigated by hand for 5 seconds, followed by 10 seconds of sonic activation, and this was repeated four times over 1 minute. Laser activation of the irrigants was also performed. Irrigant was introduced into the canal for 10 seconds, followed by 5 seconds of laser activation, (0.25W, 20 Hz) this cycle was repeated four times.

Teeth were then crushed and serial dilutions were performed to determine the number of viable bacteria (CFU/ml) remaining in the root canals. Protein assays were conducted to quantitate the amount of biofilm obtained. Samples were also taken from each treatment group and the radicular dentinal surfaces of the root canals were viewed under scanning electron microscopy (SEM).

**Results**

The root canals that were syringe irrigated with saline were the positive controls. Activation of the irrigants with either the sonic or laser instruments resulted in reduced cellular viability of *E. faecalis*. The most dramatic reduction in viability of *E. faecalis* was seen when the Er,Cr:YSGG laser was used to activate 4% sodium hypochlorite, resulting in 99.93% ± 0.14% percentage kill.

SEM analysis showed that sonic activation with saline only caused minimal disruption to the biofilm. Teeth irrigated with sodium hypochlorite showed fewer bacterial cells on the radicular dentine but was not effective in eliminating *E. faecalis* that had invaded the dentinal tubules. Laser activation of sodium hypochlorite resulted in clean dentine walls and minimal bacteria within the dentinal tubules.
Conclusion

Sonic or laser activation of an antimicrobial irrigant resulted in more effective bacterial elimination compared to hand irrigation. Compared to syringe irrigation and sonic activation of sodium hypochlorite, laser activation of sodium hypochlorite was able to effectively disinfect the root canal.
Chapter 1. Literature Review

1.1 Introduction

Pulpal necrosis and apical periodontitis are the result of invasion by bacteria and/or their by-products into the pulp and periapical tissues respectively (Dahlén et al., 1981; Sundqvist, 1992). Apical periodontitis is defined as an “inflammatory process in the periradicular tissues caused by microorganisms in the necrotic root canal” (Haapasalo et al., 2005). Thus, the primary objective of endodontic therapy is to eliminate the causative agent of disease so as to provide an environment conducive to healing. This is achieved through a series of procedures that have become part of the established protocol of root canal therapy. The steps involved include the mechanical debridement of the root canal (Byström and Sundqvist, 1981; Dalton et al., 1998), irrigation of the root canal with suitable antimicrobial agents (Byström and Sundqvist, 1983), placement of an appropriate inter-appointment medicament (Byström and Sundqvist, 1985; Shuping et al., 2000), and finally obturation of the root canal filling with a material that elicits minimal inflammatory response and entomb the bacteria not previously eliminated (Gutmann and Witherspoon, 2002). This subsequently sets the stage for post-operative healing (Byström et al., 1987). Successful root canal therapy depends critically on the management and control of pulpal space infection. While complete removal of bacteria from an infected root canal system on a consistent basis is at present still an elusive goal, the reduction in bacterial numbers is a critical step in attaining a successful treatment outcome (Shuping et al., 2000).

1.2 The microbiological basis of endodontic therapy

The first suggestion of an association between bacteria and the development of apical periodontitis dates back to 1894, when Miller first made the discovery of microorganisms within the root canal system. However, this novel finding failed to establish bacteria as the causative agent in apical periodontitis. In fact, it took another 70 years until Kakehashi et al (1965) published the findings of their landmark work, firmly establishing the role of bacteria in the aetiology of pulpal and periapical pathology. The methodology of this simple yet elegant study involved the use of gnotobiotic and conventional rats. The pulps of both groups of animals were exposed and left open to the oral environment. The latter group of animals (conventional rats) developed an inflammatory response characterised by
pulpal necrosis, abscess and apical periodontitis, while the germ-free rats experienced minimal pulpal inflammation and exhibited substantial healing of the exposure site. As such, it can be clearly seen that the absence of bacteria permitted predictable healing, while the presence of bacteria resulted in a cascade of inflammatory responses that ultimately led to the development of periapical pathology.

Subsequent studies by Sundqvist (1976) and Möller et al. (1981) corroborated the pivotal role that bacteria play in the development of apical periodontitis. Using monkey incisors, Möller et al. (1981) devitalised a total of 78 teeth under aseptic conditions. In 26 of these incisors, the access cavities were sealed immediately, while the remaining 52 teeth were left exposed to the oral environment for a few days before they were sealed. After an observation period lasting 6 months, the results showed an absence of apical pathology in the 26 teeth that were sealed immediately, while 90% of the teeth which had the access cavities left open developed apical periodontitis. In the Sundqvist (1976) study, human incisors that had experienced trauma were evaluated. All 32 incisors were intact after the trauma, and it was established that the pulps in all 32 teeth were necrotic. Radiographically, 19 of these incisors exhibited signs of apical periodontitis. Employing anaerobic culturing techniques, it was found that 95% of teeth with apical periodontitis returned a positive culture. All of this provided compelling evidence that necrotic pulpal tissue in itself does not cause apical periodontitis, but the concomitant presence of bacteria is necessary for the development of apical pathology.

Having established the causative agent of endodontic disease, root canal therapy is thus aimed at the prevention or elimination of pulp space infections and the associated inflammatory sequelae (Trope and Bergenholz, 2002). Prevention refers to the „prophylactic“ endodontic treatment of teeth with irreversibly inflamed pulps, since the infection is relatively superficial and the radicular pulp is often vital and free of bacteria (Haapasalo et al., 2005). The aim of treatment is specifically targeted at averting the spread of infection into the periradicular tissues (Haapasalo et al., 2005). However, once infection extends to the entire root canal system, inflammation of the periradicular tissues and apical periodontitis results. When this occurs, elimination or at least reduction of bacteria within the root canal is necessary so as to ensure healing of periapical tissues. The importance of bacterial elimination was highlighted by Sjögren et al. (1997), whose study showed that this played a critical role in determining success of root canal treatment. A sample of 55 single rooted teeth with necrotic pulps and radiographic evidence of apical periodontitis were treated. Bacteriological samples of the canals were taken twice, once before and another following instrumentation of the root canal and irrigation with 0.5% sodium
hypochlorite. The teeth were then obturated at that same visit and followed for 5 years. Success was judged according Strindberg’s (1956) criteria, which stipulates that the contours, width and structure of the periodontal ligament margin had to be normal or the periodontal contours could be widened around an excess of filling material for treatment to be deemed successful. The success rate of teeth which had positive cultures prior to obturation was 68%, compared with 94% for teeth that yielded a negative culture, a difference that was statistically significant. Based on these findings, the authors concurred that despite the significant reduction in bacterial load that could be achieved through instrumentation and antimicrobial irrigation, chemomechanical preparation of the root canal in itself is insufficient to completely eradicate bacteria from the root canal. As such, they recommended the placement of an antimicrobial dressing between visits so as to achieve “thorough elimination of bacteria” (Sjögren et al., 1997). However, it should be noted that healing of the periapical lesion did occur amongst the teeth that yielded positive cultures, and the authors postulated that the remaining bacteria that survived chemomechanical preparation may have been killed following entombment within the obturated root canal, or due to the inability to access nutrition (Sjögren et al., 1997). Another possibility is that the bacteria may have survived but were not able to reach the periapical tissues to cause infection (Sjögren et al., 1997), or that the numbers of bacteria were sufficiently small such the immune response was able to manage.

It is therefore possible that endodontic therapy can be successful even with bacteria still present within the root canal after chemomechanical preparation, albeit in lower numbers according to the results of the study by Sjögren et al. (1997). However, other studies have shown that no significant differences exist whether positive or negative cultures are obtained prior to obturation (Peters and Wesselink, 2002). Separating their sample size of 39 cases into 2 groups; 21 teeth would be treated in a single visit, while the remaining teeth were treated in two visits with placement of calcium hydroxide as an intracanal medicament for 4 weeks (Peters and Wesselink, 2002). The canals were sampled several times during the root canal procedure, and the teeth were followed up for a period of 4.5 years. Prior to obturation, 8 teeth returned a positive culture, 7 teeth were from the single visit group while the remaining tooth with a positive culture had been treated in two visits. Of these 8 cases, it was found that 87.5% of these teeth that had a positive culture had healed, compared with 74% of cases that healed after yielding a negative culture prior to obturation (Peters and Wesselink, 2002). Statistical analysis revealed that there was no significant difference in terms of healing. Accordingly, Peters and Wesselink (2002) stated that there was no correlation between the “healing of endodontic lesions and the presence
or absence of a positive canal culture after proper cleaning and shaping”. When the treatment outcome of the two treatment regimens was examined, a higher percentage of teeth (81%) that had been treated in a single visit had healed compared with healing in 71% of teeth which received an intracanal calcium hydroxide medicament between the two visits (Peters and Wesselink, 2002). This led the authors to question the effectiveness of placing calcium hydroxide within the canals between visits, as other authors have also found that calcium hydroxide failed to effectively render root canals sterile (Reit and Dahlén, 1988; Ørstavik et al., 1991). Despite dressing the root canals with calcium hydroxide for a period of 14 days, 23% of the canals still yielded positive cultures (Reit and Dahlén, 1988), while Ørstavik et al. (1991) found 35% of the canals had residual infection following a 7 day application of calcium hydroxide. Nonetheless, Reit and Dahlén (1988) and Ørstavik et al. (1991) still recommended the use of an interappointment intracanal medicament, a view not shared by other authors (Peters et al., 2002; Weiger et al., 2000). It should be noted, however, that saline was used as the irrigant in the study by Ørstavik et al. (1991). It is thus no wonder that the results could not replicate that of Byström et al. (1985), who employed the use of 0.5% sodium hypochlorite.

1.3 The single versus multiple visit endodontic therapy debate

There have been issues that have been raised by proponents of both camps supporting a single visit approach and those who favour the placement of an interappointment antimicrobial medicament. The advantages of single visit endodontic therapy include less time spent in the surgery, reduced cost for the patient, and reports have indicated that the amount of post-operative pain is minimal (Mulhern et al., 1982; Trope, 1991). Endodontic therapy is concerned with the control of microbial infection, and hence the question of whether elimination of bacteria can be achieved in a single visit is paramount.

It should be recognised that endodontic disease possesses different clinical presentations. In primary endodontic disease, the pulp may be vital, or the pulp may be completely necrotic and infected, and these states represent different stages of a disease process that is dynamic and distinct (Spångberg, 2001). The diseased vital pulp occurs as a result of a carious exposure and is characterised by a locally inflamed pulp with superficial infection (Spångberg, 2001). Since the microorganisms are generally limited to the pulpal wound surface, the more apical aspect of the root canal system is generally free of bacterial infection and hence can be considered sterile (Trope and Bergenholtz, 2002). The focus is
thus on the maintenance of strict asepsis to prevent microbial contamination of the radicular root canal space, so as to avert the occurrence of apical periodontitis (Trope and Bergenholtz, 2002).

The case for single visit endodontic therapy for a pulp that is irreversibly inflamed can be made due to the relative „sterility” of the radicular portion. In a study by Pekruhn (1981), 102 patients with irreversible pulpitis were randomly assigned into either single visit or multiple visit treatment groups. Amongst the 604 cases where pulpectomy and obturation of the root canal were performed in a single visit, a remarkable 98% success rate was achieved. A subsequent study was conducted by the same author in 1986 involving 1140 teeth in 918 patients (Pekruhn, 1986). Teeth were grouped in a dental problem coding system, and it could be inferred that teeth in the groups defined as “disorders of the dental pulp of carious and noncarious origin” and “intentional pulp removal for periodontal or prosthetic reasons” most likely had vital pulps or some vital pulpal tissue in the radicular portion prior to root canal therapy (Pekruhn, 1986). A total of 604 teeth were classified in both groups, the former group containing 504 teeth, while the latter group consisted of 100 teeth. The success rates were 97.8% and 99% respectively (Pekruhn, 1986).

However, when the pulp is completely necrotic and infected, the microorganisms exist both within the pulp space and in the radicular dentinal wall (Spångberg, 2001) and may survive on the external root surface. In the latter stages of the disease process, development of an apical inflammatory lesion may occur (Trope and Bergenholtz, 2002). Root canal treatment in this scenario is targeted at removal of the necrotic tissue debris and elimination of the microorganisms that have colonised the root canal system, infecting the necrotic pulp tissue remnants as well as having invaded the dentinal tubules. Controversy still surrounds the issue of single visit endodontic treatment in the management of teeth that have necrotic pulps and associated with a periapical lesion. This is due to differing opinions as to whether or not adequate microbial control can be obtained in one appointment, without the use of an intracanal medicament.

The proponents of the single visit protocol, however, argue that the placement of an intracanal medicament does not significantly improve the outcome of endodontic therapy (Penesis et al., 2008; Peters et al., 2002; Peters and Wesselink et al., 2002; Weiger et al., 2000). In fact, microbiological sampling in the study by Peters et al. (2002) showed that a 4 week dressing with calcium hydroxide not only failed to eliminate the bacteria, but the number of bacteria actually increased significantly at the second visit in comparison to the sample taken at the end of the first treatment appointment. The study evaluated 42 canals which were divided into equal numbers into two treatment groups, one group would
receive endodontic treatment in a single visit, while the other would be treated in two visits, with an interappointment medicament placed (Peters et al., 2002). Bacteriological samples of the canals were taken at baseline and at the end of preparation on the first visit, while the two visit treatment group were sampled again at the start of the second visit and before obturation. As mentioned, the number of colony forming units increased significantly between samples taken at the end of the first visit and those taken at the start of the second appointment. While only 3 root canals from the two visit treatment group yielded a positive sample at the end of the first visit, this number had increased to 15 canals at the start of the second visit (71%). This is in contrast to other published studies which have shown a decrease in bacterial numbers following the placement of calcium hydroxide (Byström et al., 1985; Shuping et al., 2000). One potential reason why this occurred in the Peters et al. (2000) study might be the way in which calcium hydroxide was placed in the canal. Despite the increase in the number of canals that yielded a positive culture, the number of colony forming units in these canals was only 0.93% of that taken at baseline. By the end of the second visit, only 2 canals harboured bacteria that could be cultured, and the number of colony forming units had become reduced significantly. The authors thus concluded that calcium hydroxide could only limit, but was not capable of preventing regrowth of bacteria within the root canal system (Peters et al., 2002).

However, it should be noted that both the Peters et al. (2002) and Peters and Wesselink (2002) studies only utilised roots with a singular canal, despite having mandibular molars in the study, as only the distal roots of these teeth were evaluated. Single canals have simpler anatomy compared with those of multi-rooted teeth, which have cul-de-sacs and other recesses which may preclude mechanical instrumentation and access to irrigants. As such, the lack of any significant differences between the two treatment protocols may have been due to the ability of chemomechanical debridement to disinfect these single canals more effectively.

Other studies have also compared the effectiveness of calcium hydroxide, but in relation to how it could potentially affect the outcome of endodontic treatment when compared against a single visit treatment protocol (Weiger et al., 2000; Peters and Wesselink, 2002). In the study by Weiger et al. (2000), the inclusion criteria stated that teeth had to have a demonstrable radiographic lesion, and both single and multi-rooted teeth were used. Thirty-six teeth were subjected to a single visit protocol, while 31 teeth were treated in two visits, with an interappointment dressing with calcium hydroxide. The teeth were reviewed at 6 months, and then annually for up to 5 years. Radiographic examination was the means to evaluate the outcome of endodontic therapy. Overall, 52
teeth were classified as having shown evidence of "complete healing", while 11 teeth showed signs of "incomplete healing" and root canal treatment had failed in 4 teeth. Out of the 52 teeth that had healed completely, 30 teeth (30/36) belonged to the single visit treatment group, while 22 teeth (22/31) had been treated in two visits. Three out of the 4 cases that failed received the single visit treatment protocol, while the remaining 1 was from the two visit treatment group. Based on the results, it was shown that the probability of treatment success for the one visit and two visit treatment were 87% and 84% respectively (Weiger et al., 2000). The authors thus concluded that the single visit treatment protocol provided healing comparable to the two visit treatment from a microbiological perspective (Weiger et al., 2000).

More recently, a randomised controlled clinical trial was conducted to compare single visit and two visit endodontic therapy by evaluating radiographic evidence of periapical healing after 1 year (Penesis et al., 2008). In contrast to the studies by Peters et al. (2002) and Peters and Wesselink (2002), this study included multi-rooted teeth in the sample. The medicament of choice in the two visit treatment protocol was calcium hydroxide mixed with 2% chlorhexidine. Among the 63 patients that were evaluated at the 12 month follow up, 33 patients received endodontic treatment in a single visit, while 30 patients were subject to the two visit protocol. The types of teeth (i.e. anterior, premolar and molar) were distributed evenly amongst the two treatment groups. Using the periapical index (PAI) to evaluate radiographic healing (Ørstavik et al., 1986), both treatment groups exhibited statistically significant differences in the PAI score at the 12 month evaluation, but there was no significant differences between the groups (Penesis et al., 2008). The single visit group had 67% of the teeth show complete healing, while 70% of the teeth in two visit treatment group healed completely at 12 months. Again, there was no significant difference between the two treatment groups. Radiographic evaluation of healing using the PAI was also employed in the study by Trope et al. (1999). At the end of the 52 week evaluation period, the results of the study showed 64% and 74% of patients showed improvements in the PAI score when they had received single visit and two visit endodontic therapy respectively (Trope et al., 1999). Despite the 10% difference in complete healing between the two treatment groups, statistical analysis revealed no significant difference.

A two part study by Kvist et al. (2004) and Molander et al. (2007) sought to compare the outcome of single and two visit endodontic treatment from both a microbiological and radiographic perspective. Teeth treated in a single visit received a 10 minute dressing with iodine potassium iodide, while the two treatment protocol involved placement of calcium...
hydroxide within the canal between appointments. In the first part of the study, a total of 96 teeth were evaluated microbiologically through sampling of the root canal (Kvist et al., 2004). Samples were taken at the start, after instrumentation of the root canal, and after medication of the root canal. All teeth types were examined in this study. Initial sampling from the root canals demonstrated the presence of bacteria in 98% of all the root canals, and following instrumentation, this number had been reduced to 62% and 64% in the single visit and two visit treatment groups, respectively. After medication with 5% iodine potassium iodide, the root canals in the single visit group had 29% of teeth with a positive culture, while 36% of the teeth that had received calcium hydroxide in the two visit treatment protocol had residual bacteria within the root canals. The differences between the groups were not statistically significant, which led the authors to conclude that the effectiveness of placing 5% iodine potassium iodide for 10 minutes in a single visit treatment protocol was similar to a two visit treatment strategy involving the placement of calcium hydroxide between appointments (Kvist et al., 2004). Curiously, care was taken to ensure that the smear layer root canals in the one-visit treatment group was removed, but this was not performed for root canals that were treated in two visits. This might have potentially affected the effectiveness of calcium hydroxide as an intracanal medicament (Haapasalo et al., 2000), and may have influenced the outcome in favour of the one visit treatment protocol. The second part of the study was aimed at following up on the teeth two years later to determine the clinical and radiographic outcome (Molander et al., 2007). The authors also sought to determine if the results from the microbiological sampling had an influence on the outcome of endodontic treatment. After the loss of some teeth during the follow-up period, a total of 89 teeth were evaluated at the end of 2 years. Based on radiographic evaluation, 65% of teeth that had been treated in one visit had healed, while 27% of these teeth were classified as uncertain. The teeth that had been dressed with calcium hydroxide experienced a greater proportion of healed lesions at 75%, while 13% of these teeth deemed uncertain (Molander et al., 2007). Statistical analysis revealed no significant differences in healing between the two treatment protocols. When the results of microbial sampling were compared against the radiographic results, it was found that 80% of teeth that had been obturated with a negative culture had healed, while only 44% of teeth that still had cultivable bacteria within the root canal system prior to obturation were classified as healed. Despite this fairly large difference, the statistical analysis led the authors to conclude that the “presence or absence of detectable microbes just before obturation did not influence the healing results at a statistically significant level” (Molander et al., 2007). While these conclusions contradicted those of Sjögren et al.
(1997), these studies have demonstrated the lack of significant difference that calcium hydroxide has on the improvement in success rates of endodontic treatment, albeit the debatable statistical data (Trope et al., 1999; Molander et al., 2007). However, if there was a means by which the number of bacteria can be reduced to the level which is achieved following placement of calcium hydroxide as an intracanal medicament, then this may provide further evidence to support single visit endodontic therapy.

1.4 Microbial root canal sampling

In light of the current available evidence, it seems that a favourable outcome with endodontic therapy can be achieved despite the detection of bacteria within the root canal system through microbial sampling prior to obturation (Peters and Wesselink, 2002; Molander et al., 2007). The concept of microbial root canal sampling and culturing is not new, and was first suggested by Onderdonk (1901) to be implemented in a clinical endodontic strategy. However, due to the complexity of the technique, it fell out of favour with many clinicans, who then proposed alternative methods, such as the use of clinical indicators such as swelling, pain, draining sinus tracts, wet canals and foul odours, to detect the presence of intracanal microorganisms (Grossman, 1950). The publication of Möller’s work in 1966 led to the resurgence of microbial sampling. His comprehensive review of the methodology provided for a systematic protocol, starting from sterilisation of the operating field to the sampling techniques with charcoal impregnated paper points and anaerobically prepared and stored sampling fluid (VMGA). The advent of state-of-the-art anaerobic culturing techniques led to the ability to identify the microorganisms that were sampled up to species level (Möller, 1966).

This method of microbial sampling, as difficult as it is technically demanding, is currently the most effective way of establishing the presence of microbes within the root canal system. However, it is not without its failings. The sample that is obtained may or may not be a true representation of the variety of microbes that are involved in the instigation and persistence of apical periodontitis, as the bacteria that have been obtained from the paper point may be those which are least tenacious and hence most easily displaced from the root canal. Moreover, there are instances when false positive or false negative samples may be obtained. The former usually occurs when a canal is sterile, but contamination of the paper point with microorganisms from the other sources, such as a non-sterile working field, occurs; while the latter arises when no microorganisms are cultivable from an infected canal. This can be due to the inability of the paper point to
sample bacteria that are residing in areas of the root canal beyond the reach of the paper point, as a result of the complex anatomy of the root, which include various accessory canals and isthmuses and various other cul de sacs (Vertucci, 1984). Bacteria that have invaded the dentinal tubules would not be disclosed through conventional sampling with paper points (Haapasalo and Ørstavik, 1987). Another reason could be the fastidious nature of some bacteria, which makes them very difficult, if not possible, to culture, or that these microorganisms may be recovered but in poor condition, thereby resulting in the absence of growth when cultured (Reit et al., 1999). Although speculative, the presence of viable but non culturable bacteria are also thought to exist within the root canal (Lleo et al., 1998).

Nonetheless, paper point sampling is arguably the most reliable technique available currently. There have even been suggestions that sampling of the root canal after chemomechanical debridement may replace long term studies using radiographs to evaluate success of treatment, thereby acting as a “surrogate endpoint” to evaluate treatment outcome (Molander et al., 2007). While this approach may potentially be „convenient“ for the patient, it should not be embarked upon without caution. In cases where a negative culture is obtained, the root canal system may not be „sterile“, but rather bacteria may be in inaccessible regions of the root canal that cannot be sampled or in low numbers that are not cultivable. The root canals that have positive cultures should be approached with more prudence, since there is potential for such bacteria remaining within the root canal system to perpetuate apical periodontitis, leading to disease that persists after endodontic treatment has been completed.

1.5 Post treatment disease

1.5.1 The prevalence of post treatment disease

Epidemiological studies have shown that the prevalence of post treatment disease to be fairly high across the population, and the trend seems to be similar in different countries and on different continents. In a survey of the Lithuanian population, the incidence of apical periodontitis in endodontically treated teeth was 35% (Sidaravicius et al., 1999). De Moor et al. (2000) investigated the prevalence of post treatment disease in a Belgian population, and found that 40.4% of root-filled teeth had radiographic evidence of apical periodontitis. The highest incidence of apical periodontitis in root-filled teeth was that of a Danish population, where the periapical status of almost 600 endodontically treated teeth
were compared in the periods from 1974 to 1975 and from 1997 to 1998 (Kirkevang et al., 2001). In both samples of the population, approximately 50% of root-filled teeth showed evidence of apical periodontitis, and the prevalence was as high as 65% when root canal treated molars were examined (Kirkevang et al., 2001). Across the Atlantic, two selected Canadian populations were evaluated and the incidence of apical periodontitis after endodontic treatment had been completed was 44% and 51% (Dugas et al., 2003).

1.5.2 The causes of post treatment disease

There are a variety of reasons for the failure of endodontic treatment leading to persistence of disease. One of the most logical reasons would be root canal treatment that has not been performed to a high standard and a poor coronal seal (Ray and Trope, 1995). In this often cited study, the authors evaluated radiographs of 1010 endodontically treated teeth. They classified endodontic therapy to be „good” when all canals were obturated to within 0 to 2 mm from the radiographic apex and no voids were present. A „good” restoration was a permanent one that “radiographically appeared sealed” (Ray and Trope, 1995). The results showed that when both the root canal treatment and restoration were „good”, 91.4% of the teeth had no radiographic evidence of periradicular inflammation. However, if the endodontic treatment and restoration were „poor”, then the number of teeth that showed absence of apical periodontitis fell to 18.1%. This meant that a significant number of teeth with poor endodontic treatment and coronal restorations had persistent endodontic disease.

The persistence of apical periodontitis following root canal treatment can also be attributed to the fact that the lesion is a radicular true cyst, which is independent of the presence of bacteria and its by-products within the root canal (Simon, 1980). As such, a true cyst is self-sustaining and is generally less likely to resolve following non-surgical root canal treatment (Nair et al., 1993; Nair 1998). A foreign body reaction may be another cause of a persisting lesion following endodontic therapy. Endogenous cholesterol crystals deposited in periapical tissues can perpetuate apical periodontitis due to a foreign body reaction at the periapical tissues (Nair et al., 1993). Exogenous materials extruded out through the apex into the periapical tissues can also result in the failure of resolution of a periapical lesion. Examples of such exogenous materials include endodontic clinical materials, such as gutta percha, (Nair et al., 1990) and certain food particles (Simon et al., 1982). The presence of infected dentine debris in the periapical lesion as a result of apical extrusion has also been implicated in the persistence of apical periodontitis (Yusuf, 1982).
Extraradicular infection is another reason for failure of root canal treatment. A study employing the use of cultivation techniques showed the presence of extraradicular bacteria when bacteriological samples were taken from periapical tissues and scrapings of the root tip or entire root tip (Tronstad et al., 1987). Gram-negative and Gram-positive obligate anaerobes and Gram-positive facultative anaerobes were cultured (Tronstad et al., 1987). Scanning electron microscopy (SEM) has also been used and images have revealed root tips denuded of cementum and the presence of a smooth, amorphous coating adjacent to the apical foramen (Tronstad et al., 1990). Present also were “scattered bacteria of different morphological types” and these were found in “aggregates or colonies” (Tronstad et al., 1990). The authors postulated that the origin of bacteria on the external root surfaces were from the root canal, and a recent study has shown, with the aid of histological sections, that almost all of the samples which had extraradicular bacteria were associated with intraradicular bacterial biofilm (Ricucci and Siqueira, 2010). Their findings suggest that the extraradicular bacteria are sustained by the bacteria within the root canal (Ricucci and Siqueira, 2010).

As such, the final and possibly most common reason for failure of root canal treatment and persistence of apical periodontitis is the presence of intraradicular bacteria that was not eradicated during the various procedures carried out in endodontic therapy (Siqueira, 2001) or due to secondary infection associated with defective coronal restorations. This can occur even in well treated teeth, as many studies have demonstrated that a significant portion of the root canal remains untouched during chemomechanical instrumentation (Peters et al., 2001; Peters et al., 2003). This is due to the complex morphology of the root canal system, with many areas that are inaccessible to the current armamentarium (Vertucci, 2005). Even in single-canal teeth with seemingly „simple” anatomy, many are long, oval shaped, and a study has shown that up to 65% of such canals remain uninstrumented (Wu and Wesselink, 2001). Many of these uninstrumented recesses also remain unfilled following obturation of the root canal (Wu and Wesselink, 2001). A recent study by Nair et al. (2005) examined the apical 3 mm of 16 mandibular molars that had the mesial root apices resected. The endodontic treatment was performed in a single visit and the apicectomy was performed on the mesial roots at the same visit. Histological examination and transmission electron microscopy (TEM) found that microorganisms were still present in the apical root canal system of 14 of the 16 root apices, constituting 88% of all specimens. Ten out of 11 root tips that had an isthmus showed presence of microorganisms within. In some of the sections, there were “multilayered microbial condensation that resembled biofilms” attached to the radicular dentinal walls. It can be
argued that since there was no sampling of the root canals and culturing of the microorganisms, the bacteria visualised on the histological sections and on TEM may not be vital. However, TEM images showed the presence of “numerous dividing forms of cocci and rods”, indicative of vital microorganisms. Also, the use of paper points to sample the root canal system may not be able to gain access to areas such as isthmuses and accessory canals. The presence of these microorganisms after the root canal treatment has the potential to perpetuate apical periodontitis, leading to post-treatment disease. As such, the question of whether single visit endodontic therapy can effectively disinfect the root canal system is still debated, despite evidence indicating success rates equalling multi visit treatment (Weiger et al., 2000; Peters and Wesselink, 2002; Peters et al., 2002; Penesis et al., 2008).

1.5.3 The microbiology of post-treatment disease

The fact that endodontic treatment can succeed despite the presence of viable bacteria following chemomechanical preparation and medicament placement is because the vast majority of the bacteria have been eliminated and the complex interactions between different bacterial species and its environment have been disturbed (Siqueira and Rôças, 2008). However, the bacteria that remain within the root canal system, and continue to survive within this nutrient-depleted, environmentally-altered condition are those that have been able to resist or escape the disinfection procedures and adapt to these new parameters. These „survivors“ are then able to perpetuate the disease process of apical periodontitis. While these bacteria may have survived the initial root canal treatment, others may have gained access into the obturated root canal system through coronal leakage, also referred to as secondary infection (Siqueira and Rôças, 2008). Nonetheless, it is recognised that the incidence of post treatment disease is significantly higher in teeth diagnosed with preoperative apical periodontitis (Sjögren et al., 1990; Marquis et al., 2006), and some authors have thus inferred that “persistent infections instead of secondary infections are the major cause” of post-treatment disease (Siqueira and Rôças, 2008).

The profile of the microbiology within the root canal of a tooth with post-treatment disease differs markedly from a tooth with primary infection. In primary apical periodontitis, the microorganisms that are found within the root canal system are those that are able to thrive in the ecological conditions of the necrotic root canal. There is a dominance of obligate anaerobes, along with the presence of some facultative anaerobes, and the genus frequently isolated include Bacteroides, Prevotella, Porphyromonas,
Campylobacter, Eubacterium, Propionibacterium, Lactobacillus, Actinomyces, Peptostreptococcus and Streptococcus (Sundqvist, 1992). The number of strains of bacteria found in the root canals with primary infection generally range between 1 and 9 (Gomes et al., 1996; Gomes et al., 2004), with one study finding an average of 5.4 strains in the root canals (Sundqvist, 1992).

In contrast, the microbiota in teeth with post-treatment disease usually consists of either one or only a few species (Molander et al., 1998; Sundqvist et al., 1998). As mentioned, within a root canal that has undergone endodontic therapy, there exist selective pressures upon the microorganisms. Currently, it is not known if this process of selection is dependent upon a specific resistance of the microorganisms to the antimicrobial agents employed or on a particular ability of some bacteria to survive in the environment of a treated root canal where nutrition and cooperative interactions with other bacteria is minimal. However, it is thought that bacteria involved in post-treatment disease have developed various strategies to persist within the root canal. Firstly, the bacteria within the root canal are organised in biofilms, which offer resistance to many of the antimicrobial agents (Distel et al., 2002). The ability of some of these bacteria to evade chemomechanical debridement contributes to their survival, by residing in anatomical irregularities and invading dentinal tubules (Haapasalo and Ørstavik, 1987; Nair et al., 2005). Moreover, some bacteria, such as Enterococcus faecalis, have the ability to resist the antimicrobial effects of medicaments such as calcium hydroxide (Byström et al., 1985). When faced with scarcity or absence of nutrients, E. faecalis is also potentially capable of entering a viable but non-cultivable state (Lleo et al., 2001), and may subsequently flourish when a nutrient source is restored (Figdor et al., 2003).

Generally, there is a predominance of Gram-positive bacteria in root canals with post-treatment disease. There are approximately equal proportions of facultative and obligate anaerobes (Molander et al., 1998; Sundqvist et al., 1998). One study found that 58% of bacterial species isolated from root canals with failed endodontic treatment were facultative anaerobes, while obligate anaerobes constituted the other 42%, and Gram-positive species comprised 80% of all bacteria cultivated (Pinheiro et al., 2003a). The continued survival and persistence of Gram-positive microorganisms may imply that these bacteria are more resistant and have superior adaptive capabilities to withstand the inhospitable environment wrought by the antimicrobial measures of endodontic therapy. Some of the more commonly isolated bacterial species include Enterococci, Actinomyces, Peptostreptococcus, Streptococcus, Lactobacilli, and the most commonly isolated fungi is Candida albicans (Sundqvist et al., 1998; Molander et al., 1998; Pinheiro et al., 2003b;
The most frequently recovered bacterial genus is *Enterococcus faecalis*, and a significant number of studies have shown *E. faecalis* in teeth with post-treatment disease to be the dominant species or even present as a monoinfection (Sundqvist *et al*., 1998; Peciulene *et al*., 2000).

In a study of 100 teeth exhibiting radiographic signs of post-treatment apical periodontitis that received non-surgical retreatment, the presence of bacteria was detected in 68 teeth (Molander *et al*., 1998). Gram-positive facultative anaerobes dominated the spectrum of bacteria isolated, and amongst the Gram-positive bacteria, *Enterococcus* was the most frequently isolated genus, which was cultured from 32 teeth and represented approximately 47% of the teeth which were culture positive (Molander *et al*., 1998). Amongst these teeth, it was noted that 20 teeth had “heavy” growth of *Enterococcus* (Molander *et al*., 1998). This trend is supported by the work of Sundqvist *et al*. (1998).

Bacteria were recovered from 24 canals from a total of 54, and of these, *E. faecalis* was isolated in 9 cases. This constituted 38% of teeth which had cultivable bacteria (Sundqvist *et al*., 1998). It was not only the most commonly recovered microorganism, it was also present within the canal as the only species (Sundqvist *et al*., 1998). The teeth were also followed up for a period of 5 years and the authors noted that the success rate for retreated teeth which had *E. faecalis* isolated from the root canals had a lower success rate of 66% compared with the overall average (75%) of teeth which had cultivable bacteria but which healed completely.

Another study by Peciulene *et al*. (2000) investigated the occurrence of *E. faecalis* in root canals of teeth undergoing root canal retreatment in a population in Lithuania. Within a sample of 25 teeth, bacteria were isolated from 20 teeth. Of the 20 teeth which had cultivable bacteria, *E. faecalis* was isolated from 14 (70%). Also, *E. faecalis* was the dominant isolate in 12 of the 14 teeth, and in 5 of these teeth, *E. faecalis* was present as a monoinfection (Peciulene *et al*., 2000). A more recent study by Pinheiro *et al*. (2003a) sought to investigate the microorganisms that were commonly isolated from root canals that had failed endodontic treatment. The sample size consisted of 30 teeth, and like all the studies previously mentioned, the teeth had radiographic evidence of apical periodontitis. A total of 24 teeth had cultivable bacteria, and *E. faecalis* was isolated from 11 canals, representing 46% of teeth with bacteria present within the canals. In 6 of the teeth, *E. faecalis* was the only species recovered. A further 60 teeth with post-treatment disease were examined in another study by Pinheiro *et al*. (2003b). Gram-positive facultative anaerobes and obligate anaerobes dominated, accounting for 83% of all species recovered.
Out of these 60 teeth, only 9 teeth had no cultivable bacteria, while 28 of the teeth had a monoinfection, representing 47%. *E. faecalis* was isolated as the single microorganism in 18 of the 28 teeth and was the most commonly encountered bacterial species recovered (Pinheiro *et al.*, 2003b). In the last few years, molecular biological techniques have been employed to aid in the identification of bacteria that are particularly fastidious or are present in such low numbers as to render them almost uncultivable. Schirrmeister *et al.* (2008) used both culture and polymerase chain reaction (PCR) to detect the presence of bacteria in endodontically treated teeth with persistent apical periodontitis; the results showed that 60% and 65% of teeth yielded microorganisms. Of the teeth that tested positive, DNA from *E. faecalis* was present in 31% of teeth (Schirrmeister *et al.*, 2007).

### 1.6 Enterococcus faecalis

#### 1.6.1 An introduction to Enterococcus faecalis

The frequent recovery of *E. faecalis* from previously treated root canals of teeth with persistent apical periodontitis is testimony to its involvement in the pathogenesis of post-treatment disease. Its”“ ability to withstand the antimicrobial measures employed in endodontic therapy allows it to survive where other bacterial species succumb, which is why it is sometimes isolated as a monoculture (Sundqvist *et al.*, 1998; Peciulene *et al.*, 2000). However, it is possible also that *E. faecalis* is the only organism isolated due to the limitations of current sampling techniques.

Bacteria belonging to the genus enterococci are common inhabitants of the human gastrointestinal and genitourinary tracts (Murray, 1990). While they do not form part of the normal oral commensal microbiota of the mouth (Aas *et al.*, 2005), Enterococci can gain access to the oral cavity since they are present in foods that are consumed raw, such as cheese and fermented sausages (Franz *et al.*, 2003). As such they are transient oral microorganisms. They are Gram positive cocci that can occur singly, in pairs, or as short chains, and are frequently elongated in the direction of the chain. Most strains are non-haemolytic and non-motile. Being facultative anaerobes, they possess the ability to grow in the presence or absence of oxygen (Rôças *et al.*, 2004). Enterococcus species live in vast quantities (105-108 colony-forming units (cfu) per gram of faeces) in the human intestinal lumen and generally do not pose any problems to their hosts. They are also present in the human female genital tract and the oral cavity, albeit in lesser numbers (Koch *et al.*, 2004).
They can rely on a multitude of substrates for energy, and these include carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and many α-keto acids (Koch et al., 2004).

The capacity of the enterococci for tolerating harsh environments ensures their ability to survive where other bacteria would not thrive. They are capable of growing despite extreme alkaline conditions (pH 9.6) and high salt concentrations, and are able to resist bile salts, detergents, heavy metals, ethanol, azide and desiccation (Tendolkar et al., 2003). Their resilience has also endowed upon them the potential to grow within the range of 10°C to 45°C, and even survive at temperatures of 60°C for 30 min (Tendolkar et al., 2003). When faced with extreme stress, they are capable of potentially entering a viable but non-culturable (VBNC) state, as mentioned previously (Figdor et al., 2003). There are currently 23 enterococci species that have been identified, and these are divided into five groups based on their interaction with mannitol, sorbose, and arginine. E. faecalis belongs to the same group as E. faecium, E.casseliflavus, E. mundtii, and E. gallinarum (Portenier et al., 2003).

1.6.2 The significance of Enterococcus faecalis in infections

The significance of the enterococci species in the medical community lies in their capacity to cause nosocomial infection. The medical importance of the enterococci far outweighs the relatively insignificant proportion (less than 1%) of the total adult human intestinal microbiota they represent. They were first recognized in the 1970s as major nosocomial pathogens causing bacteraemia, endocarditis, bacterial meningitis, infection of intra-abdominal wounds, urinary tract infections, and various other infections (Jett et al., 1994). There are the approximately 800,000 cases of enterococcal infections each year in the United States alone and estimates have placed the cost of curing these infections at around $0.5 billion. The vast majority of infection-derived clinical isolates belong to the species E. faecalis (more than 80%). While vancomycin is usually the drug of choice when dealing with such infections, especially with enterococcal urinary tract and soft tissue infections, there is an increasing occurrence of vancomycin-resistant enterococci (Edmond et al., 1996). This poses a significant problem, as standard recommendations for treatment of enterococcal infections can no longer provide optimal results. Moreover, there is also the increased risk of horizontal transfer of this resistant determinant to other vancomycin-susceptible species (Pearson, 2002).
1.6.3 *Enterococcus faecalis* and endodontic infections

*Enterococcus faecalis* is a normal inhabitant of the oral cavity. A study by Sedgley (2004) showed the presence of *E. faecalis* in oral rinse samples from patients with no history of endodontic treatment, but the prevalence of the bacteria was increased significantly when the samples were taken from patients who were receiving initial endodontic treatment, midway through treatment, and patients receiving endodontic retreatment.

Clinical studies have shown that *E. faecalis* is implicated in different forms of periradicular disease. With respects to primary endodontic infections, the prevalence of this species is higher in chronic asymptomatic periradicular lesions as compared with acute periradicular periodontitis or acute periradicular abscesses. However, they generally make up a small proportion of the initial flora, which is dominated by Gram-negative species (Sundqvist, 1992). While *E. faecalis* was detected in 18% of cases of primary endodontic infections, 67% of failed cases of endodontic treatment were found to harbour the bacteria (Rôças *et al.*, 2004). This suggests that the presence of other bacteria in a mixed consortium may have an inhibitory effect on *E. faecalis*, and harsh environmental conditions that may impair other species may actually allow for the proliferation of *E. faecalis* and not impact its survival (Rôças *et al.*, 2004). This accounts for the involvement of *E. faecalis* in persistent infections within failed root canal cases. However, the possibility that *E. faecalis* may gain entry into the root canal during treatment, between appointments or even after the conclusion of treatment, thereby resulting in secondary infection, should not be discounted. As Rôças *et al.* (2004) pointed out, the origin of *E. faecalis* infecting root-filled teeth should be investigated, so as to determine whether *E. faecalis* is a major pathogen involved with the aetiology of endodontic failures or whether it is an opportunistic coloniser that takes advantage of the environmental conditions within obturated root canals. Whichever the case may be, the common recovery of *E. faecalis* in root canals of teeth where endodontic treatment has failed is testimony to the fact that this species is intimately involved in the pathogenesis and maintenance of persistent apical periodontitis.

1.6.4 The virulence of *Enterococcus faecalis*

The virulence of *E. faecalis* is attributable to certain factors involved in disease causation, and these include lytic enzymes such as gelatinase and hyaluronidase (involved
in tissue damage), cytolysin (which is lytic towards selected mammalian cells), aggregation substance (involved in the binding to leukocytes and connective extracellular matrix), pheromones (which are small linear peptides involved in conjugative transfer of plasmids and chemoattractant for neutrophils), and lipoteichoic acid (involved in adhesion to host surfaces and stimulates cytokine production by monocytes) (Rôças et al., 2004). All these factors allow *E. faecalis* to compete with other bacterial cells and alter host responses, which causes suppression of the action of lymphocytes, potentially contributing to endodontic failure. Jett *et al.* (1994) has indicated that apart from possessing these virulence factors, *E. faecalis* is also able to share these virulence traits among species, further contributing to its ability to cause disease.

The main virulence factors are:

- aggregation substance (AS),
- cytolysin,
- gelatinase,
- enterococcal surface proteins,
- extracellular superoxide production,
- hyaluronidase,

1.6.4.1 Aggregation substance

The aggregation substance is a 37 kDa protein anchored onto the enterococci cell membrane (Muscholl *et al*., 1993). It is an adhesion encoded by pheromone-responsive plasmid, facilitating the exchange of genetic material between the recipient and the donor bacteria by allowing the two bacterial cells to come into contact (Dunny, 1990; Clewell *et al*., 1993). It is through this mechanism that certain characteristics such as antibiotic resistance and plasmid-encoded virulence factors (e.g. enterococcal cytolysin) can be transferred between *E. faecalis* strains and to other species of bacteria (Clewell, 1981). The relationship between cytolysin and aggregation substance has been shown by Chow *et al.* (1993) to be synergistic, through activation of the quorum-sensing mode of cytolysin regulation. This results in deep tissue invasion and subsequent tissue damage (Chow *et al*., 1993). Apart from adhesion to other bacterial cells, aggregation substance has also been shown to facilitate attachment of the bacteria to eukaryotic cells, such as renal tubular cells (Kreft *et al*., 1992).
Aggregation substance has also been shown to provide protection against polymorphonuclear leukocyte (PMN) or macrophage-mediated killing (Rakita et al., 1999; Süßmuth et al., 2000). Using deconvolution fluorescence microscopy, it was shown that phagosomes within PMN cells that contained bacteria expressing aggregation substance were distinctly larger compared to those phagosomes that contained bacteria that had been opsonised (Rakita et al., 1999). It was suggested that maturation of the phagosome had been affected, leading to the ability of bacteria bearing aggregation substance to resist killing by PMN (Rakita et al., 1999). As such, Rakita et al. (1999) indicated that this ability to allow for intracellular survival of \textit{E. faecalis} inside PMNs strongly suggests the role of aggregation substance as a virulence factor. In another study involving macrophages, enterococci that expressed aggregation substance were significantly more resistant to killing by macrophages (Süßmuth et al., 2000). It is postulated that aggregation substance may potentially inhibit the respiratory burst that is normally involved in macrophage phagosome killing of microorganisms (Süßmuth et al., 2000).

1.6.4.2 Cytolysin

Cytolysin is a plasmid-encoded toxin, which is produced by beta-hemolytic \textit{E. faecalis} isolates (Ike and Clewell, 1992). Previously called haemolysin, this virulence factor causes the lysis of erythrocytes, polymorphonuclear neutrophils and macrophages (Miyazaki et al., 1993). There is also evidence to suggest that cytolysin isolated from \textit{E. faecalis} belongs to a new branch of the lantibiotic family. This is a group of small secreted proteins that have been shown to possess bactericidal activity against Gram-positive bacteria such as Staphylococci, Streptococci and Propionibacteria (Schnell et al., 1988; Jett and Gilmore, 1990). On the other hand, the \textit{E. faecalis} producing cytolysin is protected from lysis by the \textit{cylI} gene product. However, the mechanism through which this occurs is unknown (Coburn et al., 1999).

Other investigations have shown that environmental factors play a role in the expression of genes that express cytolysin. A study by Haas et al. (2002) elucidated a quorum-sensing mechanism for the production of cytolysin. The way in which quorum-sensing systems function is through the production of an inducing substance that “accumulates in the environment until a threshold is reached, at which point there is a change in cellular behaviour” (Hass et al., 2002). In the case of cytolysin in \textit{Enterococcus}, the products of two regulatory genes, \textit{cylR1} and \textit{cylR2}, repress the transcription of the cytolysin structural gene. However, once the level of one of the cytolysin subunit, \textit{CylL}\textsubscript{s}”
reaches a certain threshold, de-repression occurs and expression of cytolysin thus results. There is also the possibility that environmental oxygen levels may also regulate the production of cytolysin (Day et al., 2003). The genes cylL₉ and cylLₛ encode the structural subunits of cytolysin, and have been shown to be controlled by changing oxygen conditions, with increased transcription occurring under anaerobic conditions (Day et al., 2003). This has particular relevance to endodontics considering the anaerobic conditions of the root canal system (Kayaoglu and Ørstavik, 2004).

1.6.4.3 Gelatinase

Gelatinase is an extracellular zinc-containing metalloproteinase which is capable of hydrolysing gelatine, collagen, fibrinogen, casein, hemoglobin, insulin, certain E. faecalis sex-pheromone-related peptides, and some other bioactive peptides (Mäkinen et al., 1989). Gelatinase is also produced by a variety of different mammalian cells, including inflammatory cells, epithelial cells, fibroblasts, and osteoclasts. The main function of bacterial proteases is to provide peptide nutrients to the organism. However, it is possible that proteases cause direct or indirect damage to the host tissues and are therefore classified as virulence factors. Epidemiologic studies have shown that gelatinase production by E. faecalis was detected in 45-68% of clinical isolates obtained from hospitalised patients with various infections (Coque et al., 1995; Elsner et al., 2000). This was much higher compared to 27% of faecal isolates from healthy volunteers, suggesting that gelatinase plays a role in virulence.

1.6.4.4 Surface adhesins

The Enterococcal gene esp, is responsible for encoding the large, high-molecular-weight surface protein Esp, with a protein structure that consists of multiple repeat motifs. Esp has been shown to facilitate the primary attachment and biofilm formation of E. faecalis on abiotic surfaces (Toledo-Arana et al., 2001). Strains of E. faecalis that had defective esp gene transcription were unable to form a biofilm, and as such, a genetic association between the presence of esp and ability to express surface adhesins may exist (Toleda-Arana et al., 2001). While its role in the virulence of enterococci bacteria is unclear, there is speculation that the central repeat region may serve to retract the protein from the bacterial surface to evade immune surveillance of the host (Portenier et al., 2003).
Another protein, EfaA, is also expressed by *E. faecalis*, and is encoded by the *efaA* gene. Studies involving animal models have shown that mutants that possess the *efaA* gene exhibited prolonged survival, while strains of *E. faecalis* that were lacking the *efaA* gene had reduced survival (Singh *et al*., 1998). This could potentially implicate EfaA as a virulence factor (Singh *et al*., 1998).

1.6.4.5 Extracellular superoxide production

Superoxide anion is a “highly reactive oxygen radical involved in cell and tissue damage”, and can cause damage to a variety of biological compounds, including lipids, proteins and nucleic acids (Cross *et al*., 1987; Kayaoglu and Ørstavik, 2004). While production of superoxide by neutrophils and macrophages form part of the immune defence, bacteria have also been shown to produce superoxide anions. Huycke *et al.* (1996) reported that production of superoxide was common occurrence in strains of *E. faecalis*, especially if the bacteria were associated with bacteraemia or endocarditis. Commensal isolates found in the stool of healthy subjects had significantly lower levels of extracellular superoxide (Huycke *et al*., 1996).

1.6.4.6 Hyaluronidase

The enzyme hyaluronidase degrades hyaluronic acid, thereby causing tissue damage as the consequence of its activity. Occurring commonly in nature, it is produced by a wide variety of animals, ranging from snake venom to spermatozoa (Kayaoglu and Ørstavik, 2004). Hyaluronidase causes the depolymerisation of the mucopolysaccharide moiety of connective tissues, and in doing so facilitates bacterial invasion of tissues. It is also believed that one of the functions of hyaluronidase is to provide nutrition for the bacteria, as the end products of enzyme break down are disaccharides which are transported within and subsequently metabolised within the bacterial cell (Hynes and Walton, 2000).

In a study involving 28 teeth with apical periodontitis, Hashioka *et al.* (1994) found that bacterial production of hyaluronidase was related to “subacute clinical symptoms involving percussion pain”. It is postulated that the presence of extra-radicular infection, including the presence of *E. faecalis* in periapical lesions (Sunde *et al*., 2002), may be related to the action of hyaluronidase (Kayaoglu and Ørstavik, 2004). Enzymatic breakdown of the periapical tissues may be crucial in the migration of bacteria from within the root canal into the periapical lesion (Kayaoglu and Ørstavik, 2004).
AS-48 is a 7.4 kDa peptide antibiotic that was originally isolated from *E. faecalis* S-48 (Martinez-Bueno *et al.*, 1990). It is capable of causing lysis to a wide spectrum of Gram-negative and Gram-positive bacteria (Galvez *et al.*, 1989). Its lytic action on target cells is through the generation of pores in the cytoplasmic membrane, leading to increased permeability to ions and subsequent depolarisation (Galvez *et al.*, 1991). Other studies have now demonstrated that the mechanism by which AS-48 exerts its effects on the cytoplasmic membrane of susceptible bacteria is more complex than initially thought, and that certain regions of the AS-48 peptide must be present to amplify the degree of accumulation of AS-48, thereby allowing permeation of the membrane (Jiménez *et al.*, 2005). This results in the collapse of membrane potential of the susceptible bacteria (Maqueda *et al.*, 2007).

AS-48, similar to cytolysin, is encoded by a transmissible plasmid (Martinez-Bueno *et al.*, 1990), and it has been shown that the synthesis of AS-48 is controlled at the post-transcriptional level (Fernández *et al.*, 2008). The prevalence of bacteria strains that produce AS-48 has yet to be determined, and as such, its significance as a virulence factor is still unclear, especially since there has yet to be any reports of AS-48 activity against eukaryotic cells (Jett *et al.*, 1994).

However, these factors may or may not contribute to the innate characteristics of *E. faecalis* to cause disease. For example, there is currently a dearth of studies concerning the role of hyaluronidase in enterococcal virulence, and as such, the involvement of this virulence factor in the disease process of apical periodontitis remains largely speculative (Kayaoglu and Ørstavik, 2004). Moreover, *E. faecalis* relies more upon its ability to survive and persist as a pathogen in the root canals of teeth, rather than on its virulence factors (Rôças *et al.*, 2004). This is supported by the finding that *E. faecalis* is usually involved in asymptomatic periradicular diseases, and their pathogenic nature is related more to their resistance to antimicrobial agents, rather than their high virulence. However, these virulence factors may be crucial to the survival of *E. faecalis* within the nutrient-poor environment of the endodontically-treated root canal. Cytolysin and AS-48 may be instrumental in suppressing the growth of other bacteria so as to minimise competition, and the effectiveness of these virulence factors against other Gram-positive and Gram-negative bacteria may be the reason why the number of species in a tooth with post-treatment disease is low (Kayaoglu and Ørstavik, 2004). The ability of *E. faecalis* to produce hyaluronidase may allow for enzymatic degradation of hyaluronan, which is present in
dentine, thereby obtaining nutrition from the scarce resources within the root canal (Kayaoglu and Ørstavik, 2004).

1.6.5 The survival of *Enterococcus faecalis* within the root canal

The ability of *E. faecalis* to invade the dentinal tubules enhances its ability to evade the antimicrobial agents employed in endodontic therapy and thus improve its chances of survival. In 1964, Shovelton conducted histological examination of 97 extracted teeth that had been diagnosed clinically as non-vital. It was evident that bacteria had invaded the dentinal tubules in 61 of these teeth. Chronic infection generally yielded a greater degree of bacterial penetration of dentinal tubules, and the invasion of the tubules did not occur immediately after bacterial invasion of the root canal system (Shovelton, 1964). Also, the bacterial penetration seemed to be fairly random in that the number of tubules that contained bacteria was highly variable between different teeth and between different sections of the same tooth (Shovelton, 1964). Also variable was the depth of penetration, the presence of sclerotic tubules physically hinders the penetration of bacteria into the tubules, and variation in the depth of bacterial invasion is due to the differences in tubule diameters (Love, 1996). The presence of an intact cementum layer also determines the degree of penetration, since its absence permits a greater degree of bacterial invasion from the pulpal side (Haapasalo and Ørstavik, 1987). *In vitro* studies have demonstrated the ability of *E. faecalis* to penetrate dentinal tubules at distances of 300-400 μm, with the front of the infection reaching up to 800-1000 μm, and the depth of penetration was shown to increase with time of incubation (Haapasalo and Ørstavik, 1987). The capacity for facultative anaerobes to invade dentinal tubules had been shown earlier by Akpata and Blechman (1982), who used *Streptococcus sanguis* and *E. faecalis*. However, the obligate anerobes used in the study, *Bacteroides melaninogenicus* ss. *Melaninogenicus* and *Peptococcus asaccharolyticus*, both failed to show any evidence of dentinal tubule penetration (Akpata and Blechman, 1982). The extent of penetration of the dentinal tubules was related to the period of incubation, with *E. faecalis* exhibiting a greater degree of invasion after 3 weeks compared to *S. sanguis*; nonetheless, both “thick masses” of these bacteria could be visualised in histological section “abutting” the radicular dentinal walls on the pulpal side (Akpata and Blechman, 1982). It was also found that the cervical aspect of the tooth root showed a greater amount of dentinal tubule penetration compared with the apical third (Akpata and Blechman, 1982), which is likely to be due to the differences in tubule diameters and distribution of dentinal tubules in different parts of the tooth (Love,
In an in-vitro model similar to that of Haapasalo and Ørstavik (1987), Siqueira et al. (1996) used scanning electron microscopy to investigate the ability of certain commonly isolated microorganisms to invade dentinal tubules. Dentine cylinders were infected with Porphyromonas endodontalis, Fusobacterium nucleatum, Actinomyces israelii, Porphyromonas gingivalis, Propionibacterium acnes and E. faecalis. The former 5 strains of bacteria used were obligate anaerobes, and E. faecalis being the only facultative aerobe. Only E. faecalis, P. acnes, and A. israelii invaded the dentinal tubules, while P. gingivalis, P. endodontalis and F. nucleatum showed limited success in penetrating the dentinal tubules, although when P. gingivalis invaded dentinal tubules, they travelled to great depths (Siqueira et al., 1996). This supports other studies showing that E. faecalis is able to penetrate dentinal tubules (Akpata and Blechman, 1982; Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990; Siqueira et al., 1996). However, it should be noted that apart from the Akpata and Blechman (1982) study which used human teeth, Haapasalo and Ørstavik (1987), Ørstavik and Haapasalo (1990) and Siqueira et al. (1996) used bovine dentine cylinders, the justification being that dentinal tubules in bovine teeth were similar in size, morphology, and density to human teeth. Nonetheless, the ability to penetrate the dentinal tubules is an effective strategy in evading the antimicrobial measures taken during root canal therapy. As shown in the Haapasalo and Ørstavik (1987) and Ørstavik and Haapasalo (1990) studies, calcium hydroxide was unable to effectively eradicate E. faecalis despite the placement of the medicament within the lumen of the dentine cylinder for a period of 10 days. Live bacteria could be cultivated from the circumferential dentine within the first 100 μm, indicating the relative ineffectiveness of calcium hydroxide to exert its bactericidal effects once the E. faecalis had penetrated within the dentinal tubules (Haapasalo and Ørstavik, 1987). In addition, the inherent pH buffering ability of dentine and the poor diffusion of hydroxyl ions into the dentine significantly reduces the antibacterial properties of calcium hydroxide (Haapasalo et al., 2000). A pH decrease from 12.5 to 11.5 has been shown to allow for an increase in survival of E. faecalis (Byström et al., 1985). Camphorated paramonochlorophenol (CMCP), in both liquid and gaseous forms, was shown to be more effective at eradicating E. faecalis from the depths of dentinal tubules (Haapasalo and Ørstavik, 1987; Ørstavik and Haapasalo, 1990). However, Messer and Chen (1984) had previously shown that CMCP was extremely volatile, and up to 95% of it was lost within the first 24 hours which would negatively impact its antibacterial capabilities. Moreover, the dentine cylinder model employed in the studies by Haapasalo and Ørstavik (1987) and Ørstavik and Haapasalo (1990) allowed for maximum contact of the radicular dentine with the medicament. In an in-vivo situation, the
anatomical complexity of the root canal system would result in the inability of the medicament to reach some areas.

The capacity of *E. faecalis* to endure prolonged periods of starvation until an adequate nutritional supply becomes available accounts for their persistence in obturated root canals where nutritive sources are scarce. *E. faecalis* cells have been shown to be able to reside within dentinal tubules and remain viable within obturated canals for up to 12 months (Sedgley *et al.*, 2005). The starved cells are able to recover by using serum as a nutritional source when it becomes available, and is derived from the surrounding tissues including bone and periodontal ligament (Love, 2001). The availability of serum results in the “recovery and resumption of growth of *E. faecalis*”, as the bacteria are capable of growth in the presence of 50% serum for more than 4 months (Figdor *et al.*, 2003). Even when the concentration of serum was reduced to 1%, sustained growth of a small number of *E. faecalis* occurred (Figdor *et al.*, 2003). Serum has also been shown to aid in the binding of *E. faecalis* to type I collagen, and there may be a synergistic effect between serum and *E. faecalis*, such that the invasion of the dentinal tubules by *E. faecalis* is promoted by the presence of serum (Love, 2001).

When challenged with starvation within the root canal environment, *E. faecalis* may not be able to survive if it did not enter a state of dormancy. It is believed that *E. faecalis* is capable of entering a viable but nonculturable state (VBNC), which is a survival strategy when faced with environmental stress (Lleo *et al.*, 1998). The VBNC state is characterised by failure of the bacteria to grow on routine bacteriological media, but the cells are still alive and demonstrate active metabolism, albeit at very low levels (Lleo, 1998; Oliver, 2005). As such, these bacterial cells escape detection when conventional microbiological tests are applied. Once resuscitated, cells are capable of returning to a culturable state (Oliver, 2005). In a study by Lleo *et al.* (2001) monitoring the transcription of *pbp5* mRNA, *E. faecalis* was shown to be capable of persisting in the VBNC state for up to 3 months.

However, the subject of the VBNC state is controversial, as several investigators have argued against its existence (Bogosian *et al.*, 1998). Bogosian *et al.* (1998) used a mixed culture recovery method which involves mixing two easily distinguishable strains of culturable and non culturable cells together, and then applying various resuscitation techniques to determine whether the culturable and non culturable cells respond. This way, the cells that are resuscitated from a nonculturable state can be distinguished from those that were merely residual culturable cells (Bogosian *et al.*, 1998). Their results showed the nonculturable cells were dead, while only the culturable strain were recovered, such that
the resuscitation that was meant to occur from the VBNC state was due to the growth of the remaining culturable cells (Bogosian et al., 1998). A more recent study has recently investigated the protein expression of *E. faecalis* cells growing under severe nutrient limitation, to distinguish them from that of those cells that are in the VBNC state (Heim et al., 2002). It was found that the protein profile of VBNC cells differed from those that were starved, and it was concluded that the VBNC state is a “distinct physiological phase within the life cycle of *E. faecalis*” (Heim et al., 2002).

Apart from the ability to invade dentinal tubules and endure starvation, *E. faecalis* is also able to form biofilms. A biofilm is defined as microbial communities that are attached to a surface substrate, whether it is organic or inorganic (Svensäter and Bergenholtz, 2004). Extracellular proteins and polysaccharides are produced to facilitate the initial attachment of the microorganisms and to hold the various species of bacteria together within the biofilm, constituting up to 85% of the volume of the biofilm (Svensäter and Bergenholtz, 2004; Portenier et al., 2003). There are different stages in the formation of the biofilm. In summary, it begins with the adsorption of macromolecules that are present in bacteria that are in the planktonic phase to the substrate surface. This results in the formation of the conditioning film, consisting of secreted microbial products (Bushner et al., 2000). This conditioning film is responsible for the selection of various planktonic phase microorganisms which attach, thereby influencing the composition of bacteria within the biofilm (Bushner et al., 2000). This sets the stage for the next phase, which involves the adhesion and co-adhesion of selected microorganisms. The early microbial colonisers are responsible for exerting selective pressures on subsequent co-adhesion of other microorganisms (Kolenbrander et al., 2002). The third and final stage involves the maturation of the biofilm through the multiplication of attached microorganisms, leading to a “structurally organised mixed microbial community”. The innate attributes of the microorganisms as well as the architecture and nature of the environment within the biofilm determine the growth and succession of microorganisms that reside within the biofilm (Svensäter and Bergenholtz, 2004). While the biofilm structure has long been established in dental plaque research, it was first described in the infected root canal by Nair (1987). His study involved the use of transmission electron microscopy (TEM) to examine the root canals of 31 teeth. He noted dense aggregates adhering to the radicular dentinal walls, with bacteria embedded within an extracellular matrix of amorphous material (Nair, 1987).

There are several advantages to being a part of a biofilm. One of the benefits of being a part of a large microbial community is the ability break down large macromolecules,
such as glycoproteins, for nutrition through the combination of various enzymes secreted by the different bacteria (Marsh and Bowden, 2000). However, it is currently not known if such a cooperative relationship exists for bacteria within the biofilm in the root canal system (Svensäter and Bergenholtz, 2004). It has also been shown that different bacteria within the biofilm are capable of cell-to-cell communication, a mechanism known as quorum sensing. While research into this area first began with the expression of bioluminescence in marine symbiotic bacteria (Engebrecht et al., 1983), it is now known that bacteria within a biofilm are also capable of being involved within a signalling network. Communication between bacteria is mediated by diffusible molecules known as autoinducers that accumulate in the external environment. Within the biofilm, acylated-homoserine lactone (HSL) molecules act as the autoinducer and HSL have been shown to play a critical role in the development of bacterial biofilms (Davies et al., 1998). Mutant bacteria that were not capable of autoinducer production produced thinner biofilms compared to bacteria that had normal HSL production (Davies et al., 1998). Apart from biofilm formation, quorum sensing is also important in the function and survival of bacteria within the biofilm.

Bacteria within a biofilm have been shown to be up to 1000 fold more resistant to antimicrobial agents (Gilbert et al., 1997). In the case of *E. faecalis*, it has been shown to be resistant to vancomycin at a concentration 4 times that of the minimum inhibitory concentration (MIC) when grown as a biofilm on cellulose filters compared to the planktonic state (Foley and Gilbert, 1997). The increased resistance to vancomycin as a result of being in a biofilm was confirmed when cells derived from the biofilm were regrown in planktonic cultures and exhibited similar susceptibility to vancomycin as the original cells. The authors thus suggested that the increased resistance of *E. faecalis* to vancomycin was due to the expression of a different “biofilm” phenotype compared to the planktonic phenotype (Foley and Gilbert, 1997). It is also thought that the environment within the biofilm may account for the decreased effectiveness of antimicrobial agents. The anaerobic conditions within the biofilm reduce the efficacy of antibacterial agents such as aminoglycosides, which function optimally under aerobic circumstances (Tack and Sabath, 1985). The lowered pH due to the accumulation of acidic metabolic waste products may also dull the effectiveness of certain antibiotics (Portenier et al., 2003). Another reason for the reduced effectiveness of antibiotics in a lower pH environment may be because the bacteria enter a non-growing state as a result of the accumulation of metabolic waste. Since antibiotics such as penicillin affect the cell wall synthesis of bacteria, non-
growing cells would not be affected by the antibiotics, thus rendering the antibiotic ineffective (Tuomanen et al., 1986).

Another reason for the increased resistance to antimicrobial agents is the reduced penetration through the biofilm, as shown by Suci et al. (1994) who found that even a relatively thin biofilm (15 to 30 μm) could significantly impede transport of ciprofloxacin through a biofilm composed of *Pseudomonas aeruginosa* cells. However, calculations made by Stewart (1996) suggest that while “transport limitations may impinge on the efficacy of selected antibiotics when used against biofilm infections”, other mechanisms may result in the reduced efficacy of antimicrobial agents against biofilm infections, such as the possibility that phenotypically altered or slow growing bacteria within the biofilm are less susceptible to the effects of the antimicrobial agents. Deactivation of the antimicrobial agent may also occur, for example, enzymes such as β-lactamase can inactivate ampicillin (Anderl et al., 2000).

The ability of *E. faecalis* to survive and subsequently persist in the root canal stems from its resistance to various medicaments, such as calcium hydroxide. In a study by Distel et al., 2002), *E. faecalis* managed to grow within the root canals of extracted single-canal teeth despite the presence of calcium hydroxide within the canal, both as a paste and as a medicated point. The latter group required only 2 days before all the root canal samples were colonised by *E. faecalis*, while prolonged exposure of the root canal sample to *E. faecalis* in the former group resulted in the development of a biofilm (Distel et al., 2002). Scanning confocal laser microscopy revealed *E. faecalis* colonies on the dentine surface and the surface of the calcium hydroxide dressing after an incubation period of 86 days, while another root canal that had been inoculated for 160 days showed the presence of mushroom shaped colonies characteristic of a well-developed biofilm (Distel et al., 2002).

Previously, it was thought that production of certain proteins in response to environmental stress might aid in the resistance of *E. faecalis* to the effects of calcium hydroxide. However, the use of chloramphenicol to block protein synthesis in *E. faecalis* revealed that this has no impact on cell survival, indicating that stress-induced protein production is not important for the survival of *E. faecalis* at high pH (Flahaut et al., 1997). The induction of tolerance to high pH was independent of protein synthesis because pre-treatment in the presence of chloramphenicol did not alter cell survival.

A study by Evans et al. (2002) sought to investigate the resistance mechanisms employed by *E. faecalis* to resist the effects of calcium hydroxide and revealed that *E. faecalis* is resistant to killing by calcium hydroxide at or below pH 11.1. There are several mechanisms that were found to be at play. Firstly, *E. faecalis* is able to maintain pH
homeostasis, in which the internal pH is kept within a narrow range so that enzymes and proteins can maintain normal function. This is achieved through the buffering ability of the cytoplasm and the low cell membrane permeability to ions (Booth, 1985). Secondly, the proton pump that the *E. faecalis* possesses is capable of driving protons into the cell to acidify the cytoplasm, thereby lowering the internal pH in the presence of an external environment that has an elevated pH due to calcium hydroxide. When the proton pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used to inhibit its function, a 20-fold reduction in *E. faecalis* survival resulted after 30 minutes of exposure to high pH (Evans *et al*., 2002). The effectiveness of this stress response, however, is not without limits. When overwhelmed, it is unable to maintain cellular viability when the pH reaches 11.5 and above (McHugh *et al*., 2004). However, root dentine has the tendency to buffer the high pH of calcium hydroxide, and thus, it is unlikely that a pH of 11.5 can be maintained within the dentinal tubules (Tronstad *et al*., 1981). Nerwich *et al*. (1993) had shown that the environment within the radicular dentine dressed with calcium hydroxide may reach a pH of only about 10.3, well within the tolerance of *E. faecalis*. In addition, Haapasalo *et al*. (2000) illustrated the inhibitory effect of dentine on various root canal medicaments including calcium hydroxide, sodium hypochlorite, chlorhexidine, and iodine potassium iodide. The inactivation of the antibacterial effects of these medicaments may be attributed to the diverse components of dentin including dentin matrix, type-I collagen, hydroxyapatite, and serum (Portenier *et al*., 2001).

### 1.7 Endodontic therapy

#### 1.7.1 The importance of chemomechanical debridement

The absence of a functioning circulatory system in the root canal system following bacterial infection and pulpal necrosis dictates that conventional measures for the elimination of infection, i.e. the host defence system and systemic antibiotic therapy, will not be effective. As such, predictable eradication of the bacterial infection requires physical disruption and removal of the microorganisms. Mechanical instrumentation is one of the primary means of bacterial reduction in endodontic treatment, provided it achieves the following goals:

1. Removal of vital and necrotic tissue from the main root canal(s);
2. Creation of sufficient space for irrigation and medication;
3. Preservation of the integrity and location of the apical root canal anatomy; and
4. Establishment of a convenience form to facilitate root canal filling (Hulsmann et al., 2005).

The effectiveness of mechanical instrumentation alone in the elimination of bacteria was studied by Byström and Sundqvist in 1981. Using then state-of-the-art anaerobic bacteriological techniques, they were able to demonstrate that mechanical debridement of the canal, while using only sterile saline as an irrigant, was capable of reducing the number of microorganisms by 100-1000 fold. However, despite the multiple appointments, none of the teeth were rendered bacteria-free. Subsequent studies using contemporary endodontic techniques and rotary nickel-titanium (Ni-Ti) instruments confirmed these findings. Dalton et al. (1998) assessed the difference between traditional stainless steel files and 0.04 taper engine-driven Ni-Ti files, while following Byström and Sundqvist’s (1981) protocol of using only sterile saline as an irrigant. Again, it was not possible to ensure complete eradication of the bacteria predictably, as only 28% of the canals were rendered bacteria-free. However, the number of bacteria was reduced significantly when the canal was instrumented to larger sizes. These results supported the results of earlier studies, such as that by Ørstavik et al. (1991), who were able to demonstrate that instrumentation of the apical canal up to a size #45 file was capable of diminishing the bacterial growth by 10-fold. Other investigators published results which corroborated these findings, but despite the significant reduction in bacterial numbers with increasing apical enlargement, complete elimination remained elusive (Siqueira et al., 1997; Wu and Wesselink, 1995). Card et al. (2002) did, however, record 100% elimination of bacteria in single canal canines and premolars following a single instrumentation visit, with the apical aspect instrumented to approximately size #60 (#8 size Profile series 29 instrument). Root canals of multi-rooted teeth, however, did not register similar success. About 89% to 93% of the canals of these molars were bacteria-free following enlargement with bigger file sizes, a difference that was significant from those that were not prepared to such large apical sizes (Card et al., 2002).

The recognition that mechanical debridement suffered severe limitations in the complete elimination of bacteria led to the use of irrigants in conjunction to facilitate removal of tissue remnants, dentine debris and bacteria. Coupled with irrigants possessing anti-bacterial properties, bacteria killing could be enhanced, especially in areas inaccessible to mechanical debridement (Trope and Bergenholtz, 2002). This was illustrated by Cvek et al. (1976), who compared the antibacterial effect of chemomechanical preparation of root canals in permanent non-vital maxillary incisors with immature apices with those with mature roots. The experimental sample comprised of
three groups of teeth, which were mechanically debrided and irrigated with either sterile saline, 0.5% or 5.0% sodium hypochlorite solutions. Sampling of the root canals were conducted at two points during the experiment: initially after removal of necrotic tissue; and later, after completion of chemomechanical preparation. The results clearly showcased the limited effectiveness of an irrigant without anti-bacterial properties; the group of teeth irrigated with sterile saline still had significant numbers of bacteria, especially those with immature apices. The groups of teeth that were irrigated with either 0.5% or 5% sodium hypochlorite exhibited a suppression of bacterial numbers. The classic study by Byström and Sundqvist (1983) compared the antibacterial properties of 0.5% sodium hypochlorite and sterile saline irrigants in infected root canals, and the results of their study substantiated the findings of Cvek et al. (1976). The use of sodium hypochlorite irrigation coupled with mechanical instrumentation rendered 80% of the canals bacteria-free after the fifth and final appointment, while saline irrigation was only capable of only 53%. Again, despite the combination of mechanical debridement and irrigation with anti-bacterial irrigants, attaining complete eradication of these endodontic microorganisms was unachievable. Even studies that utilised contemporary rotary endodontic files, in conjunction with sodium hypochlorite as an irrigant, failed in the quest for complete eradication. Shuping et al. (2000) reported on the ability to decrease numbers of intra-canal bacteria with increased apical instrumentation sizes. The supplementary use of 1.25% sodium hypochlorite resulted in 61.9% of the canals being bacteria-free canals. However, the use of calcium hydroxide as an intra-canal medicament increased this figure to 92.5% of the canals (Shuping et al., 2000). Clearly, these studies illustrate the inability to achieve perfectly bacteria-free canals with chemomechanical preparation. Nonetheless, their capacity to reduce bacterial numbers is significant in the quest to eradicate infection.

1.7.2 The complex anatomy - Preventing disinfection of the root canal system

The hard tissue repository of the dental pulp takes on a multitude of configurations and shapes. The root canal system comprises of 2 portions, the pulp chamber, situated in the anatomic crown of tooth, and the root canal, located in the anatomic root. The root canal begins with funnel-shaped canal orifices, which end at the apical foramen, which open onto the root surface between 0.5 to 3 mm from the centre of the root apex (Kuttler, 1955; Vertucci 1984). Factors such as physiologic aging, pathology and occlusion can alter the configuration of the root canal system through the production of secondary and tertiary dentine and cementum. The complexity of its anatomy is further enhanced by the presence
of lateral, accessory and furcation canals, intercanal connections, apical deltas and apical foramina (Vertucci, 2005).

Apart from varying canal configurations, canals often also have different shapes in cross section, and difficulties have been noted in the cleaning and obturation of these canals (Wu and Wesselink, 2001). An investigation into the preparation and obturation of oval canals by Wu and Wesselink (2001) found that 40% of such canals could not be adequately instrumented. This finding was echoed in the study by Peters et al. (2001). Using micro-computed tomography (micro-CT), they compared the effects of four preparation techniques on root canal volume and surface area using three-dimensionally reconstructed root canals in extracted teeth. Their results showed that at least 35% of the surface area of root canals was left untouched regardless of the instrumentation technique (Peters et al., 2001).

Accessory and lateral canals are extensions from the pulp to the periodontium, the former being any branch of the main pulp chamber or canal that communicates with the external surface of the root, while the latter is an horizontally extending accessory canal that is located within the root (American Association of Endodontists, 2003). These lateral canals occur mainly in the apical third of the root canal, accounting for 73.5%, while the incidence in the middle and cervical third of the root is 11.4% and 6.3% respectively (Vertucci, 1984). Developmentally, these accessory and lateral canals are formed as a result of the entrapment of periodontal vessels in Hertwig’s epithelial root sheath during calcification (Cutright and Bhaskar 1969). These canals are often difficult, if not completely impossible, to instrument. As such, predictable obturation of these canals is similarly difficult.

As mentioned, mechanical instrumentation has its limitations. Hence, there is a need to ensure that areas within the root canal anatomy that cannot be accessed by files be rendered clean and disinfected. Irrigation with an antimicrobial solution is thus important in the elimination of microorganisms from the infected root canal. It is thus no wonder that much of contemporary research credits shaping and cleaning of the root canal as the most significant factor in the prevention and treatment of endodontic disease (Haapasalo et al., 2005).

1.7.3 The solution – Sodium Hypochlorite

Through the history of endodontics, there have been a myriad of solutions used as irrigants. Ideally, an irrigant should have a broad antimicrobial spectrum, efficacious
against microorganisms that are have become organised in a biofilm. It should also be able to prevent formation of the smear layer, and remove it following instrumentation. Also, in a perfect world, the irrigant should be able to dissolve necrotic pulp tissue but be non-toxic to periradicular tissues and non-allergenic (Zehnder, 2006). Unfortunately, such an irrigant is not available to us. To date, sodium hypochlorite is the most widely used endodontic irrigant in contemporary endodontic practice, and is arguably still the best irrigating solution, since it fulfils most of the criteria stipulated for an ideal irrigant. However, the use of sodium hypochlorite is associated with potential dangers due to its cytotoxicity, as it has the ability to cause necrosis, haemolysis and skin ulceration (Pashley et al., 1985). Swelling, extraoral ecchymosis and ulceration of the oral soft tissues have been reported following extrusion of sodium hypochlorite (Linn and Messer, 1993, Hulsmann and Hahn, 2000). Severe pain and paresthesia of the involved area are some of the symptoms experienced by patients (Witton et al., 2005).

Sodium hypochlorite is non-specific and is capable of killing a wide spectrum of microorganisms, ranging from bacteria to fungi and viruses (Zehnder, 2006). The effects of sodium hypochlorite on necrotic tissue are due to the hypertonicity of the solution. The ability to oxidise, hydrolyse and osmotically draw fluids out of tissues makes it a potent proteolytic solvent (Pashley et al., 1985). The mechanism through which sodium hypochlorite exerts its bactericidal effects has never been shown experimentally, and is believed to be brought about by the oxidative action of the undissociated hypochlorous acid on the sulphhydryl groups of bacterial enzymes. The inhibition of these enzymes result in interference with metabolic functions, which then presumably result in the death of these bacteria (Dychdala, 1991).

One of the most compelling reasons to use sodium hypochlorite is its strong antibacterial properties. In a study by Spratt et al. (2001), the anti-bacterial capabilities of different irrigants were tested against single-species biofilms of Prevotella intermedia, Peptostreptococcus micros, Streptococcus intermedius, Fusobacterium nucleatum and Enterococcus faecalis cultivated on membrane filter discs. Amongst the field of irrigants, which included 5 ppm colloidal silver, 2.25% sodium hypochlorite, 0.2% chlorhexidine and 10% iodine, it was found that sodium hypochlorite was found to be the most effective irrigant, especially against E. faecalis, P. intermedia and P. micros, capable of achieving 100% kills at 15 min and 60 min exposure (Spratt et al., 2001). Sodium hypochlorite proved to be somewhat less effective with S. intermedius and F. nucleatum after 15 min of exposure, but was still able to achieve 100% kill at the longer time interval. It should be noted, however, that the biofilm had been incubated for only 48 hrs and were grown on
cellulose nitrate membrane filters placed on agar plates, which thus allowed for maximal exposure to the irrigant. Gomes et al. (2001) tested the effectiveness of several concentrations of sodium hypochlorite in the elimination of *E. faecalis* grown up as colonies on agar plates. The results showed that sodium hypochlorite was able to kill *E. faecalis*, but the time taken to kill the bacteria was inversely proportional to the concentration of sodium hypochlorite. Full strength sodium hypochlorite (5.25%) was able to kill *E. faecalis* in less than 30 secs, while a concentration of 0.5% required 30 min for complete eradication of *E. faecalis*. A more recent study by Vianna et al. (2007), employing the same study design as that Gomes et al. (2001), investigated the effectiveness of sodium hypochlorite at concentrations, ranging from 0.5% to 5.25%, against black-pigmented gram negative anaerobic bacteria and facultative anaerobic microorganisms. Sodium hypochlorite at all concentrations, even the lowest, was capable of eliminating the obligate anaerobes (consisting of *Porphyromonas endodontalis*, *Porphyromonas gingivalis* and *Prevotella intermedia*) in 15 secs. The facultative anaerobic microorganisms, comprising *Staphylococcus aureus*, *Candida albicans* and *E. faecalis*, required longer periods of time, which were again inversely related to the concentration of sodium hypochlorite used. This shows the greater resistance of facultative anaerobes to antimicrobial agents. Nonetheless, the maximum concentration of 5.25% of sodium hypochlorite was able to kill the facultative anaerobes in 15 secs. Another *in vitro* study by Oliveira et al. (2007), unlike those mentioned above, attempted to replicate the *in vivo* scenario by using extracted premolar root canals. The canals were inoculated with *E. faecalis* for 7 days, and paper points were used to sample the root canals. Three microbiological samples were taken from each root canal, before chemomechanical preparation, immediately after chemomechanical preparation and a final one 7 days after completion of chemomechanical preparation. Two concentrations of sodium hypochlorite were tested, 1.5% and 5.25%. Both these concentrations reduced the number of colony forming units (CFU) significantly immediately after chemomechanical preparation, with the higher concentration resulting in 100% eradication of *E. faecalis*. However, while the 5.25% sodium hypochlorite was able to maintain the lower number of CFU after 7 days, there was a greater increase in CFU in the group irrigated with 1.5% sodium hypochlorite, although the difference was not statistically significant (Oliveira et al., 2007). Nonetheless, the common vein amongst these studies is that the effectiveness of the antimicrobial action of sodium hypochlorite is directly proportional to its concentration.

While this seems to be the trend for *in vitro* studies, it has not been shown to be true when extrapolated to *in vivo* studies. In fact, an *in vitro* study by Siqueira et al. (2000)
which investigated the effectiveness of three different concentrations of sodium hypochlorite of 1%, 2.5% and 5.25% found increasing concentrations of sodium hypochlorite resulted in greater reduction of *E. faecalis* from the root canals of extracted premolars, but the difference was not statistically significant. The authors suggested that the organic material consumed available chlorine, thus reducing the antibacterial activity of sodium hypochlorite. While higher concentrations of sodium hypochlorite may have a higher level of reserve of chlorine, irrigation with copious amounts of a weaker sodium hypochlorite solution with frequent replenishing may maintain a high enough level of available chlorine to eradicate bacteria from the root canal (Siqueira *et al*., 2000). The classic clinical study by Byström and Sundqvist (1983) showed the relative efficacy of sodium hypochlorite at the low concentration of 0.5% compared to saline. No bacteria could be recovered from 12 out of 15 teeth despite the absence of an intracanal medicament between appointments, only 8 of the 15 teeth had no recoverable bacteria when irrigated with saline (Byström and Sundqvist, 1983). It was the opinion of the authors that 0.5% sodium hypochlorite was the “most desirable concentration” as it provided maximal antimicrobial effect while the toxicity to the periradicular tissues was minimal (Byström and Sundqvist, 1983). However, it should be noted that the teeth received treatment over 5 appointments, and hence the results may not be completely applicable especially if the root canal was completed in a single visit. Results from a subsequent clinical study by Byström and Sundqvist in 1985 validated their earlier opinion when their study found no significant differences between 0.5% and 5% concentrations of sodium hypochlorite in terms of the antibacterial action. The work by Siqueira *et al*. (2000) seems to corroborate the evidence provided by the two Byström and Sundqvist studies (1983, 1985). However, it should be noted that *E. faecalis* was the bacteria used in the study by Siqueira *et al*. (2000), while the bacterial population in the Byström and Sundqvist studies (1983, 1985) are characteristic of a primary infection, which generally are more susceptible to the antibacterial effects of sodium hypochlorite. The Gram-positive facultative anaerobe, *E. faecalis*, is often isolated in cases of persistent infection, and is generally more resistant. Another point of note is that *E. faecalis* had been inoculated within the root canal and incubated for only 24 hrs in the Siqueira *et al*. (2000) study. As such, an established biofilm may not be present, rendering the *E. faecalis* susceptible to even the lower concentrations of sodium hypochlorite. This point was subsequently addressed in a more recent study by Berber *et al*. (2006), which examined the effects of three different concentrations of sodium hypochlorite on extracted premolars, similar to the samples used by Siqueira *et al*. (2000). However, the teeth were inoculated with *E. faecalis*
and incubated for 21 days to allow establishment of a biofilm. Bacterial penetration of the
dentinal tubules was also confirmed with scanning electron microscopy (SEM). Their
results showed that the highest concentration of sodium hypochlorite at 5.25% was most
effective at eradicating *E. faecalis* from the root canal. Apart from microbiological
sampling with paper points, Berber *et al.* (2006) also used burs of increasing diameter sizes
to obtain dentine samples of various cross sectional depths of radicular dentine. Again,
sodium hypochlorite at a concentration of 5.25% was most effective at eliminating *E.
faecalis* that had penetrated into the dentinal tubules. The next most effective concentration
was 2.5%, followed by 0.5%. However, despite the greater effectiveness of the higher
concentrations, the differences between the three were not statistically significant (Berber
*et al.*, 2006).

The ability of sodium hypochlorite to eliminate bacteria from the dentinal tubules of
radicular dentine has been the subject of several investigations. Zou *et al.* (2010) evaluated
the effect of concentration, time of exposure and temperature on the penetration of sodium
hypochlorite into dentinal tubules using extracted human anterior teeth. The crowns and
apical thirds of the roots were removed, and the remaining root section stained with crystal
violet. The root sections were treated with varying concentrations of sodium hypochlorite
(1%, 2%, 4% and 6%) for 2 min, 5 min and 20 min at different temperatures. The results
revealed that the greatest penetration into dentinal tubules (300 μm) was achieved with the
highest concentration of sodium hypochlorite, incubated for 20 min at the highest
temperature of 45ºC (Zou *et al.*, 2010). Despite this result, it should be noted that the use of
dye does not mimic the true *in vivo* situation, and bacteria such as *E. faecalis* have been shown to be capable of penetrating up to 800-1000 μm along the length of the dentinal
tubules (Haapasalo and Ørstavik, 1987). Another recent *in vitro* study sought to determine
the concentration of sodium hypochlorite and irrigation time required to disinfect bovine
dentine cylinders inoculated with *E. faecalis* for a period of 3 weeks (Retamozo *et al.*, 2010). Again, different concentrations of sodium hypochlorite were tested and applied for
different time intervals. The results showed once again that the highest concentration of
sodium hypochlorite at 5.25% applied for a period of 40 min resulted in the most effective
disinfection of the dentine cylinder. Treatment with the lower concentrations of 1.3% and
2.5% sodium hypochlorite was not able to effectively eradicate the infection. While the
results of the study indicate that *E. faecalis* was not completely eradicated with the use of
the lower concentrations, it did not quantify the number of colony forming units of *E.
faecalis* that had remained following treatment with the lower concentrations of 1.3% and
2.5% sodium hypochlorite since the dentine cylinders were placed into a tube of broth and
the presence of absence of turbidity used as the only means to determine the presence of remaining viable bacteria. Even if a small number of bacteria were remaining, placing it in a nutrient-rich broth would cause it to multiply and grow. Moreover, irrigating with sodium hypochlorite for a period of 40 min may not be completely practical in a clinical setting. To date, the optimal time for any given concentration of sodium hypochlorite to exert its antimicrobial effects has yet to be determined in randomised controlled in vivo studies. Consideration into this area should be given since the advent of rotary endodontic instruments has significantly reduced treatment time (Peters, 2004), and subsequently reduced the amount of time that sodium hypochlorite is within the root canal system. Nonetheless, this study clearly illustrates the ability of *E. faecalis* to withstand the antimicrobial effects of sodium hypochlorite.

The resistance of *E. faecalis* is further emphasised in a clinical study by Peciuliene et al. (2001), which sought to determine the effect of instrumentation and the antimicrobial effect of irrigation with sodium hypochlorite in previously endodontically treated teeth with persistent apical periodontitis. To prevent chloroform from having a negative effect on the viability of the microorganisms, all root filling material was removed with hand instruments. Two microbiological samples were taken, the first one prior to instrumentation and irrigation with 2.5% sodium hypochlorite, the second was taken immediately after the completion of chemomechanical preparation, which was completed at the same appointment. Microbiological samples taken prior to chemomechanical preparation revealed the presence of bacteria in 33 of the 40 root canals; amongst them *E. faecalis* was detected in 21 canals. Following chemomechanical preparation, *E. faecalis* was still isolated from 6 root canals, a testament of the persistence and resistance of these bacteria to sodium hypochlorite. Unfortunately, the study only used a single concentration of sodium hypochlorite, which would otherwise have aided in determining if higher concentrations of sodium hypochlorite would result in more effective eradication of *E. faecalis*.

From the aforementioned studies, it is clear that there are a multitude of factors that determine the effectiveness of sodium hypochlorite as an antimicrobial irrigant, namely concentration, time of exposure, the volume used and the rate of exchange, and possibly temperature. However, it is clear that the eradication of bacteria involved in post-treatment endodontic infections, such as *E. faecalis*, are extremely persistent and may require more extreme measures to ensure their eradication.
1.7.4 Irrigation - The limitations of current techniques

The traditional use of the syringe and needle irrigation technique has stood the test of time, but the limitations are immediately apparent. The physical flushing action from this technique is weak, and the complex anatomy severely limits the ability of the irrigant to penetrate within the confines of the root canal system. Much of the literature has consistently shown that syringe irrigation, when used in combination with mechanical instrumentation, fails to thoroughly eliminate the microbial population within infected root canals.

There are a number of variables, such as the depth of placement of the needle, the diameter of the needle, that determine the effectiveness of the flushing action (Chow, 1983; Teplitsky et al., 1987). Ram’s study (1977) showed that when syringe irrigation is used, the irrigant is unable to reach more than 1 mm beyond the tip of the needle. To counter the limited penetration of irrigant, some believed that the solution lay in increasing the volume of irrigant. However, studies have shown that an increase in volume does little to improve the flushing action (van der Sluis et al., 2006). The efficacy of removal of dentinal debris is not significantly improved either; however, the improvement is somewhat more pronounced in canals where the apical third have been instrumented to larger apical sizes (Abou-Rass and Piccinino, 1982). This view is substantiated by more contemporary studies. An in-vitro study showed that instrumentation of the mesiobuccal canals of mandibular molars to an apical size #50 could significantly reduce the amount of intracanal bacteria compared with size #35 (Rollison et al., 2002). Plotino et al (2007) recommended the use of needles with smaller diameters (30 gauge) to facilitate penetration of the irrigant into the apical depths of the root canal. Studies detailing the effectiveness of the use of such needles are still lacking, and as such, no firm conclusions can be made as yet.

1.8 Acoustic energy – Sonic and ultrasonic

1.8.1 The early years

Ultrasound refers to sound energy that possesses a frequency beyond the range of human hearing, which is 20 kHz. Ultrasonic instruments were introduced into the field of endodontics by Richman in 1957, but it took almost two decades before Martin (1976) experimented with the utilisation of acoustic energy from an ultrasonic instrument to
activate irrigants. The device employed in this study was essentially a direct adaptation of the ultrasonic scaler utilising the phenomenon of magnetostriction, which involves the conversion of electromagnetic energy to mechanical energy, to produce the characteristic oscillatory action of a file. His study involved the inoculation of molar root canals with four different strains of bacteria, organisms that were frequently cultured from root canals during endodontic therapy. The aim was to test the quantitative bactericidal effect of the ultrasonically generated acoustic energy, as well as the potential additive effect when antibacterial irrigants were used. The results showed that the ultrasonic activation of a neutral buffered solution, in itself, possessed bactericidal effects as a consequence of its “physical biologic acoustic action”, but this had an “effective plateau after 4 to 5 minutes” (Martin, 1976). When the acoustic energy was used in conjunction with anti-bacterial agents, there was a synergistic effect which led to more “effective destruction of the test organisms” (Martin, 1976). The results led Martin to believe that “ultrasonic lavage” would aid in enabling irrigants to penetrate into various complexities of the root canal system. The other advantage of combining ultrasonic energy with antibacterial agents was the chemical aspect, which involved bonding, dissociation effects, activation of radicals and oxidation of the irrigant used. As seemingly devastating as the physical effects of ultrasonics were, which was postulated to lead to the rupturing of bacterial cell walls and a “scrubbing and cleaning” mechanism, Martin prudently added that ultrasonics alone would be “insufficient for good bacterial reduction”, and strongly recommended the use of irrigants with bactericidal properties.

A few years later, K-type files were attached to an ultrasonic instrument to facilitate the instrumentation of the root canal (Martin et al., 1980). When this occurred, the file is converted into a velocity transformer, which is defined as “a mechanical device designed to match the impedance of the transducer to that of the load” (Martin et al., 1980). The way in which this is achieved is by funnelling the vibrations through a tapered structure, which was in the form of a K-type file with a conical linear taper (Martin et al., 1980). When compared against hand instrumentation of root canal dentine, the ultrasonically powered K-type file was found to be superior in terms of total dentine removed (Martin et al., 1980). It was believed that the ultrasound behaved in accordance with Huygen’s principle, which stated that “every vibrating point on a wave front is regarded as the centre of a new disturbance”, and that collective effect constitutes a new wave form, and as such, this propagating oscillating wave caused the file to be drawn through the canal (Martin et al., 1980). As a result of their work, Martin and Cunningham (1984, 1985) subsequently
coined the term „endosonics”, and this was defined as the “ultrasonic synergistic system” of root canal instrumentation and disinfection.

The popularity of this instrumentation technique soon soared, with a surge of publications in this field. The clinical effectiveness of endosonics was gauged through histological (Cunningham et al., 1982a; Langeland et al., 1985), bacteriological (Martin, 1976; Cunningham et al., 1982b) and microscopic techniques (Martin et al., 1980; Cunningham and Martin, 1982; Cameron, 1983). When the endosonic method was compared against hand instrumentation, the majority of publications highlighted the superiority of the former technique, in terms of its efficiency in the mechanical preparation of the root canal (Martin et al., 1980; Nehammer and Stock, 1985). However, there were other studies that found no significant differences in the efficacy of both endosonic and hand techniques (Weller et al., 1980). Some investigators even showed that conventional hand instrumentation was superior to the ultrasonic device in the elimination of bacteria from the root canal (Barnett et al., 1985).

Evolution of these „endosonic” devices soon followed, with the development of low-frequency ultrasonic units that operated between 1 to 8 kHz (von Arx et al., 1998; von Arx and Kurt, 1999). The advantage of these units in terms of instrumentation was that the shear stresses produced were lower, and hence caused lesser alteration to the tooth surface (Layton et al., 1996). However, results of studies investigating the effectiveness of ultrasonic and sonic activation of files to mechanically prepare the root canal system are generally divided and contradictory. The inability of these instruments to live up to expectations was due to its relative inefficiency, and this is due to the restrictive environment of the narrow, uninstrumented root canal. This constraint severely limits the oscillatory motion of the file, thereby limiting its effectiveness (Walmsley et al., 1989).

Much of the early work focused on studying the effectiveness of the „endosonic” technique at that time, and there was initially a paucity of literature on the biophysical aspects of ultrasonic employed in endodontics (Walmsley, 1987). Subsequently, studies which investigated the underlying mechanisms of the ultrasonic phenomenon in endodontics started to emerge.

1.8.2 Activation of irrigants – The new wave

Since Martin’s (1976) early study, there is now renewed interest in the role of ultrasonics in enhancing the flushing action of irrigants (Druttman and Stock, 1989; Cheung and Stock, 1992). It has been shown to be a useful adjunct in improving the
efficacy of irrigation solutions in the removal of organic and inorganic debris from the root canal system (Weller et al., 1980). Ultrasonic activated irrigation, or passive ultrasonic irrigation (as van der Sluis et al. (2007) prefers), was first described by Weller et al. (1980), in reference to the fact that unlike earlier uses of the ultrasonic instrument in endodontics, the file attached to the ultrasonic device does not actively engage the root canal dentine, and hence is not involved in mechanical instrumentation of the root canal. Rather, ultrasonic activated irrigation is dependent on the acoustic energy transmitted from the oscillating file, driven by an ultrasonic device, to an irrigant introduced into the root canal (van der Sluis, 2007). This results in the irrigant travelling at higher velocity and an increased volume of flow and exchange of irrigant (Lee et al., 2004a). The energy derived from the ultrasonic device is capable of inducing acoustic streaming and cavitation of the irrigant (Ahmad et al., 1987a; Ahmad et al., 1987b; Ahmad et al., 1988; Lumley et al., 1991; Ahmad et al., 1992; Roy et al., 1994). This would facilitate the movement of irrigants into anatomical areas of the root canal not normally accessible with syringe irrigation, such as in webs, fins and anastomoses, since the active streaming of the irrigant enhances its potential to contact a greater surface area of the canal wall (Baumgartner and Cuenin, 1992). Other researchers have also shown that ultrasonic activated irrigation may enhance the tissue-dissolving capability of solutions with a good wetting ability, especially if the pulp tissue remnants or smear layer are wetted completely by the appropriate irrigant solution (Moorer and Wesselink, 1982).

1.8.3 Acoustic streaming

Acoustic streaming is defined as the “the rapid movement of fluid in a circular or vortex-like motion around a vibrating file” (Walmsley, 1987). Within the root canal system, the acoustic streaming that occurs is known as acoustic microstreaming, which refers to the streaming which arises from the frictional forces between a boundary and a medium carrying vibrations of circular frequency (Leighton, 1994). The boundary is in reference to the obstacles that are within the sound field of the ultrasonic instrument, and this leads to complex steady-state or non-oscillatory streaming patterns formed close to the wire. This streaming pattern is not random; it corresponds to the characteristic pattern of nodes and antinodes along the length of the oscillating file. The nodes refer to the area of the file where the oscillation is minimal, while the greatest oscillation occurs at the antinodes (Walmsley, 1987). This is illustrated in figure 1, where N denotes the nodes, and A refers to the antinodes.
A significant amount of research has been undertaken, especially in the late 1980’s to early 1990’s, to investigate the phenomenon of acoustic microstreaming surrounding the use of sonic and ultrasonic instruments. Ahmad et al. (1987a) were able to elucidate the streaming pattern that arose as a result of acoustic streaming. It was observed that liquid that was in close proximity to the surface of the file was transported from the apical end to the coronal end of the file. It was also noted that an irregular arrangement of eddy currents occurred adjacent to the file, converged mainly around the apical aspect of the file. The eddy current around the tip of the file also appeared to be more rapid as compared to the flow of the eddy current in the more coronal aspect of the file. In a subsequent paper, Ahmad et al. (1987b) reported that the acoustic streaming resulted in a distinctive pattern of currents. There was a primary field, which comprised of rapidly moving eddy currents in which the “fluid element oscillates about a mean position”, and the existence of a “superimposed secondary field” which consisted of patterns that flowed a relatively slower velocity. The flow in this secondary field was said to be “time independent” (Ahmad et al., 1987b). When the streaming patterns were studied, the bulk of the eddy currents were found to be arranged in groups of four along the length of the file, with the greatest number of eddy currents concentrated around the tip of the file (Ahmad et al., 1987b). It was also noted that the smaller sized Cavi-endo files (sizes #15 and #20) generated a greater number of clusters of eddy currents compared with the larger files (size #25). Despite the fewer number of clusters, the pattern of distribution along the length of the larger file was comparable to that of the smaller files.
Apart from the above features, closer examination of the individual eddy currents within the primary field revealed that the direction of rotation of the fluid within each eddy current was opposite to that of the adjacent eddy current (Ahmad et al., 1997). In the secondary field, the flow pattern was longitudinal and symmetrical along both sides of the activated file, and it was evident that movement of fluid was from the apical end of the file towards the coronal aspect. It was also noted that there were flow patterns near the tip of the file that were directed apically, and the streaming velocities of the eddy currents at the tip of the file were much more rapid compared with those located more coronally (Ahmad et al., 1987). Interestingly, the velocity of the eddy currents are related inversely to the size of the file used; the streaming was slower when the larger size #25 file was used as compared to the smaller sizes #15 and #20 (Ahmad et al., 1987). When power settings were increased, there were no increases in the number of eddy currents observed, although the size of the eddy currents and the velocity of those currents were noted to be increased. Figure 2 shows the acoustic streaming of a file immersed in water, while figure 3 is an illustration of the primary and secondary fields, with the directions of the eddy currents indicated.

Figure 2. Acoustic streaming of a file oscillating in water (van der Sluis et al., 2007).
Figure 3. Diagrammatic representation of the acoustic streaming around a file (van der Sluis et al., 2007).

Taken together, it was deduced that the velocity of the acoustic streaming would be affected by a number of factors, namely the position of the eddy current along the length of the file, the power setting on the ultrasonic unit, and finally, the size of the file (Ahmad et al., 1997b). The displacement amplitude changes proportionately with the power setting of the ultrasonic unit. This has been expressed in the following equation:

\[ v = \frac{\omega \varepsilon_0^2}{a} \]

where \( v \) is the velocity of the acoustic streaming, \( \omega \) is \( 2\pi \) times the driving frequency, \( \varepsilon_0 \) is the displacement amplitude of the tip of the file, and \( a \) is the radius of the file (Ahmad et al., 1987b). Based on the equation, more vigorous acoustic streaming can be achieved by utilising a “freely vibrating file of a small size subjected to high power setting”, that is through employing a higher frequency, and having greater displacement amplitude of the apical aspect of the file. This equation is based upon the use of a file that is tapered and of circular cross section (Ahmad et al., 1987b). Thus, in accordance with the above equation, the velocity of acoustic streaming generated by a sonic instrument can be assumed to be lower due to its lower driving frequency.

Acoustic streaming brings about fluid motion, which results in shear stresses on the “boundaries in the ultrasonic field”. In the case of root canal treatment, this would refer to
the radicular walls of the root canal. As such, the generation of hydrodynamic shear stress would be the dominant physical mechanism by which the surface of the root canal walls are cleaned when ultrasonic activated irrigation is applied (Roy et al., 1994). The amount of hydrodynamic shear stress is proportional to the streaming velocity, which occurs at its maximum at the tip of the ultrasonically activated instrument. As such, it is reasonable to assume that the hydrodynamic shear stress would be concentrated in the vicinity of the tip, which most likely would be positioned closer to the apical aspect of the root canal. The progressive taper of an instrumented canal could also be a potential aid, since the smaller surface area of the apical aspect of the root canal would be subjected to increased energy from the acoustic streaming. This would result in the concentration of hydrodynamic stresses at the apical end of the canal, thereby facilitating the removal of debris from the root canal walls (Ahmad et al., 1987b). The explanation for the increased effectiveness in more confined areas is the stronger forces generated by the primary streaming currents, which are located in closer proximity to the surface of the vibrating instrument. The increased speed of the primary streaming, as compared to the secondary streaming currents, accounts for the greater hydrodynamic stresses (Ahmad et al., 1987b). The caveat is that the tip must be freely vibrating at this part of the root canal, not dampened by contact with the root canal wall, and as such, the file is not used as a device to mechanically debride the canal (Ahmad et al., 1987b).

There are studies outside of the field of dentistry that have discovered the physical and biological effects of hydrodynamic shear stress. Williams (1972) found that acoustic streaming was capable of generating hydrodynamic shear stresses capable of disrupting biological cells. Other researchers have found that these shear stresses are of sufficient intensity to remove the mucopolysaccharide surface coatings from mammalian cells (Martinez Palomo, 1970).

1.8.5 The effectiveness of sonic and ultrasonic activated irrigation

Investigations into the effectiveness of ultrasonic activation of irrigants date back to the 1980's. Many of the studies have attempted to assess the ability of this irrigation technique in the removal of pulpal remnants and dentinal debris (Cameron, 1987; Lee et al., 2004a; Gutarts et al., 2005), smear layer (Cameron, 1983) and planktonic bacteria (Sjögren and Sundqvist, 1987; Huque et al., 1998; Weber et al., 2003). Amongst the earliest studies was that by Ahmad et al. (1987b), which compared the cleanliness of root canals following instrumentation with ultrasonically activated files with those that
underwent ultrasonic activation of irrigants. All the teeth in both experimental groups were mechanically debrided in a similar manner, i.e. with ultrasonically activated size #15 files. However, one group was additionally subject to ultrasonic irrigation for 5 minutes, where an ultrasonic activated file was inserted to within 1 mm of the apex and allowed to oscillate freely. With the aid of scanning electron microscopy, the results revealed that the canals which had the ultrasonically activated file oscillating freely within the canal were consistently cleaner. There was virtually no smear layer in the apical third of canals that underwent ultrasonic irrigation, an unsurprising result considering the finding that the greatest velocity of acoustic microstreaming, and hence hydrodynamic shear stresses, are generated at the most apical aspect of an ultrasonically activated file (Ahmad et al., 1987a). Their results place the effectiveness of ultrasonic irrigation in favourable light, given that ethylenediamine tetracetic acid (EDTA) was not used as an irrigant, and a lower concentration of sodium hypochlorite was employed in the group which had the irrigant (1% sodium hypochlorite) ultrasonically activated, compared to the 2.5% sodium hypochlorite used for the other group. In a recent study by Lee et al. (2004), grooves and depressions were cut into instrumented canine root canals to simulate uninstrumented canal irregularities. These grooves and depressions were filled with dentinal debris and subject to ultrasonic irrigation with 2% sodium hypochlorite for 3 min. This was compared against conventional syringe irrigation, and the results showed that ultrasonic irrigation was superior in the removal of debris from the simulated canal irregularities. In some of the specimens, there was complete removal of the debris from the grooves and depressions. While the creation of such „irregularities” may be somewhat artificial, the advantage is that the amount of debris at baseline prior to irrigation is known, and as such, comparison of the amount of debris removal by each irrigation protocol can potentially be more accurate.

Given the relatively simple anatomy of the teeth examined in the aforementioned studies (Ahmad et al., 1987b, Lee et al., 2004), other investigators have utilised curved molar canals (Jensen et al., 1999). Generally, the greater complexity of such canal anatomy would hamper the complete debridement of the root canal system. In this study, the curved molar root canals, numbering 60 in total, were subjected to hand instrumentation up to an apical size of #35. Comparing syringe irrigation against sonic and ultrasonic activated irrigation, it was found that the latter two methods were significantly superior to syringe irrigation when 5.25% sodium hypochlorite was used as the irrigant. It was noted that there was no difference between the sonic and ultrasonic activation, with the ultrasonic method scoring slightly higher in terms of remaining debris. While this is surprising, since the acoustic microstreaming generated by the sonic unit is of lower intensity compared with
that of ultrasonic, the investigators did not employ the use of a scanning electron microscope (SEM). Instead, photomicrographs with a transparent grid were used to assess the amount of debris remaining in the root canal. Despite the relatively rudimentary assessment technique, the effectiveness of irrigant activation in producing cleaner canals is evident.

Another study which compared sonic and ultrasonic activation of irrigants found no significant difference between the two instruments (de Gregorio et al., 2009). The sonic instrument used in the study is the EndoActivator®, and had recently been introduced into the market (Ruddle, 2007). It is claimed that the “vigorous intracanal fluid agitation” occurs though both “acoustic streaming and cavitation” (Ruddle, 2007). The phenomenon of acoustic microstreaming and the resultant hydrodynamic shear stresses is based on the assumption that flow of the fluid occurs around the file without becoming “detached” from the surface of the file (Roy et al., 1994). However, when the displacement amplitude reaches a certain threshold and the negative acoustic pressure amplitude attains a critical value, the flow can separate from the surface of the file, “forming a wake which results in bubble formation”. This process is known as hydrodynamic cavitation (Ahmad et al., 1988; Roy et al., 1994). Given that the EndoActivator® operates at a maximum of 10,000 cycles per minute only (166 Hz), it is doubtful that the phenomenon of cavitation is at work here. The low frequency generated by the sonic instrument is too low, and below the threshold necessary for cavitation to occur (Ahmad et al., 1987a). The claim that cavitation occurs with the EndoActivator® is also disputed by Jiang et al. (2009), whose group used a high speed camera to record the oscillation of the polymer tip within a glass root canal model at 4000 frames per second. Not only was there no cavitation observed, but their results also contradicted those of de Gregorio et al. (2009). Using a similar model to that in the Lee et al. (2004a) study, they found that the ultrasonic instrument, which was capable of generating a driving frequency of 30 kHz, caused greater removal of dentinal debris from grooves made in the root canal wall compared with the EndoActivator®.

All of the aforementioned studies occurred under in vitro conditions. Also, ultrasonic irrigation occurred for 3 min in both the Jensen et al. (1999) and Lee et al. (2004) studies, while in the Ahmad et al. (1987b) study, ultrasonic irrigation was implemented for 5 min within each canal. While this would ensure that there would be sufficient time for the activation of irrigants to cause more thorough debridement, it would also translate into a significant amount of clinical time added to the preparation of a molar. Moreover, not all studies have found ultrasonic activation of irrigants to be more effective. Mayer et al. (2002) found that while ultrasonic activation of the irrigants used in their study, namely
sodium hypochlorite and EDTA, did produce slightly cleaner canals as compared with the non-activated controls, the difference was not statistically significant. It should be noted that in their study, the irrigating time was 1 min, compared with the longer irrigation times mentioned in the above studies. This timing, the authors felt, was more “clinically practical”. The results in the study by Abbott et al. (1991) also revealed no significant difference between canals that were subject to ultrasonic activation of the irrigants, and other groups which had the irrigant introduced with a syringe. The study examined a variety of irrigants, with a total of 6 treatment groups, and used SEM to evaluate the results. The volume of irrigants used was 18 ml per group, which is more than the volume used in the previously mentioned studies. It has been shown that the volume of irrigant that is used to flush the root canal system can influence the quality of root canal debridement (Yamada, 1983). It was shown that when higher volumes of sodium hypochlorite and EDTA were used to irrigate the root canal system, significantly cleaner canals were obtained as compared with smaller volumes (Yamada, 1983). The high volumes used in the Abbott et al. (1991) study may explain why there were no significant differences in canal cleanliness between those canals irrigated with the syringe and those where ultrasonic activation was applied.

As such, a combination of an adequate volume of irrigant, type of irrigant and the application of acoustic energy can bring about effective cleaning and disinfection of the root canal system. Despite the fact that the use of acoustic energy to activate irrigants is not a recent introduction to endodontics, it has yet to be established as an essential part of the protocol for root canal treatment. The potential of this “old school” technology, employed in a new fashion, is immense and should be harnessed to enhance the predictability of root canal disinfection and improving the success rate of endodontic therapy.

1.9 Lasers

In 1960, Maiman developed the world’s first laser (Kimura et al., 2000). A laser is defined as “a device which transforms light of various frequencies into a chromatic radiation in the visible, infrared, and ultraviolet regions with all the waves in phase capable of mobilizing immense heat and power when focused at close range” (Kimura et al., 2000). Traditionally, lasers are classified according to the physical construction of the laser, i.e. whether the laser is gas, liquid, solid state, or semiconductor diode, and the type of medium which undergoes lasing (e.g. erbium, chromium:yttrium-scandium-gallium-garnet (Er,Cr:YSGG) laser) (Walsh, 2003). Lasers have also been classified according to the
potential hazards, when in contact with skin or exposure to the eye (Walsh, 2003). An exhaustive list of the different types of lasers is beyond the scope of this review. Suffice to say, the potential that the power of laser possessed led to the exploration of its use in the field of dentistry (Goldman et al., 1964; Stern and Sognnaes, 1964). Early studies were conducted on the use of the ruby laser, which Maiman (1960) had developed, on caries by studying its effects on subsurface demineralisation. Since then, the list of applications has expanded, encompassing the various aspects of dentistry.

1.9.1 Lasers in endodontics

The first reported use of lasers in endodontics was published by Weichman and Johnson (1971). In their in vitro study, their attempt to seal the apical foramen with an infrared (CO\(_2\)) laser was met with failure. Despite the less than desired result, tremendous interest was generated and has led to a body of research into the potential applications of lasers in the field of endodontics.

The promise and potential of lasers in removal of dental hard tissue have also led to the exploration of its use in the instrumentation of the root canal. A recent in vitro study sought to compare the efficacy of the Er,Cr:YSGG laser to that of „traditional” rotary nickel titanium files (Radatti et al., 2006). The manner in which the laser would instrument the canal is known as hydrokinetic debridement, which is described as a process of “removing biologic material through the use of high speed water spray” (Eversole et al., 1997). The results of the study showed that the canals that were treated with the Er,Cr:YSGG laser resulted in significantly more debris than the canals that had been instrumented with rotary nickel-titanium files (Radatti et al., 2006). Moreover, the rotary instruments were more efficient, as the laser powered hydrokinetic debridement required twice as much time to perform (Radatti et al., 2006). Other problems included the ledging and other irregularities of the canals with the laser that resulted in blockage of the canal, while apical patency was maintained for all samples instrumented with the rotary nickel-titanium files (Radatti et al., 2006). The authors thus suggested that the laser be used as an “adjunct to rotary instrumentation”, potentially in conjunction with an antimicrobial irrigant such as sodium hypochlorite (Radatti et al., 2006).
1.9.2 Disinfection of the root canal system with lasers

One of the areas of greatest interest in endodontic research remains the elimination of bacteria within the root canal system. It would almost seem natural that the lasers also be employed in the disinfection of the root canal.

1.9.2.1 Photo activated disinfection

Amongst the multitude of methods to employ this technology to aid in eradicating microorganisms within the root canal was in the form of photo-activated disinfection. Photo-activated disinfection is defined as “a method of disinfecting or sterilising a hard tissue or soft tissue site by topically applying a photosensitising compound to the site, and then irradiating this with laser light at a wavelength absorbed by the photosensitising compound, so as to destroy microbes at the site” (Wilson and Wilson, 1997). Compared with direct killing with laser, low power lasers are employed to drive the photochemical reactions, and thus reduced the tendency of direct laser energy to cause cracking, cratering and carbonisation of the dentinal walls of the root canal (Anic et al., 1998). The laser light that is used in conjunction with the photosensitiser usually emits light from the middle red portion of the visible spectrum, since this wavelength penetrates dentine to the greatest extent, as well as any blood products that are present (Odor et al., 1996). While the low power laser energy used in this technique is generally not sufficient to kill bacteria per se, certain Gram-negative anaerobic bacteria may be particularly susceptible to laser light emitted in the middle red wavelengths (Lee et al., 2004b). The photochemical reactions resulting from interaction between the absorption of the laser light by the photosensitising compound would then lead to the production of singlet oxygen, free radicals, and other reactive oxygen species (Walsh, 1997). The photosensitiser molecule is elevated to a high-energy state when it is irradiated by light of a specific wavelength, and this then leads to transference of energy to an oxygen molecule. This generates singlet oxygen and other reactive oxygen species (Walsh, 1997), which are highly lethal to practically all species of bacteria that are present in the oral cavity (Donson and Wilson, 1992; Wilson et al., 1992).

There are generally two pathways by which “photodynamic microbial damage” occurs (Ochsner, 1997). In the Type I pathway, the photosensitiser triplet state causes the transfer of electrons to molecules. The free radicals which result from these electron transfer reactions then combine with oxygen to produce cytotoxic species of oxygen. These cytotoxic species, such as superoxide, hydroxyl and lipid-derived radicals, are thereby
capable of causing damage to the bacterial cell membrane, acting against the membrane phospholipids, enzymes and receptors (Oschner, 1997). The alternate action, known as the Type II pathway, occurs when the photosensitiser triplet state transfers electrons to molecular oxygen to produce singlet oxygen. This highly excited and reactive state is capable of oxidation of proteins, nucleic acids and lipids, and is hence cytotoxic to bacteria (Oschner, 1997). The absence of resistance to these extremely reactive molecules, and the short half-life of these molecules, ensure that not only is the cytotoxic effect highly effective against microorganisms, but their actions are also localised (Lee et al., 2004b).

Photo-activated disinfection has the potential to be highly effective in eradicating the bacteria in primary root canal infections. The dominance of obligate anaerobic species, such as *Fusobacterium*, *Prevotella*, and *Prophyromonas*, in primary infection of the root canal system translates into susceptibility to killing by either Type I or Type II pathways, since Gram-negative anerobes, both obligate and facultative, are extremely sensitive to the toxic effects of oxygen compounds. This is due to the absence of catalase and other protective enzyme systems in these bacteria (Lee et al., 2004b). The incorporation of porphyrin-like molecules within the cellular structure of Gram-negative bacteria also renders them vulnerable to photo-activated disinfection, as these molecules function as endogenous photosensitisers, which are activated by particular wavelengths of visible red light (Konig et al., 2000). While effective against Gram-negative bacteria,Gram-positive bacteria may not be as vulnerable to photo-activated disinfection, since most species lack endogenous photosensitising compounds, making them quite resistant to killing by visible red light alone. The use of an appropriate photosensitiser dye, such as tolonium chloride, would allow killing of most, if not all, species of bacteria, provided binding to the cell membranes of the bacteria occurs (Silbert et al., 2000).

The effectiveness of photo-activated disinfection may thus be limited against bacteria that persist within root canals following root canal treatment, leading to failure of the endodontic therapy. The microbiota found in such cases of secondary or persistent root canal infection generally tends to be Gram-positive facultative bacteria (Sundqvist et al., 1998). While studies have shown that the addition of an appropriate photosensitiser dye may render them susceptible to killing by photo-activated disinfection, these bacteria must be in direct contact with the dye so that binding of the dye to their membranes can occur (Lee et al., 2004b). The presence of bubbles within the photosensitiser dye solution may also interfere with the binding to the bacterial cell membranes (Lee et al., 2004b). Experiments have thus been conducted using planktonic cultures of *Streptococcus mutans* and *Enterococcus faecalis*, and the results showed that a 120 second exposure to photo-
activated disinfection, employing such dyes as methylene blue or tolouim chloride, is capable of killing 97 - 99.9% of the bacteria present in the culture (Silbert et al., 2000). Moshonov et al. (1995) investigated the use of neodymium:yttrium, aluminium, garnet (Nd:YAG) laser in combination with a photosensitiser dye, nigrosin, on the eradication of E. faecalis. Irradiation of the canals was conducted for 2 minutes at 4.5W and results were compared against positive and negative controls, as well as exposure to 1% sodium hypochlorite for 2 minutes. While the Nd:YAG laser, when used in conjunction with the photosensitiser dye, eliminated more than 99% of the bacteria culture, complete disinfection of the root canals did not occur. In contrast, 1% sodium hypochlorite was able to eliminate all of the bacteria, and no bacteria could be detected on their cultures and scanning electron microscope images (Moshonov et al., 1995). The results of this study reflect poorly upon the effectiveness of photo-activated disinfection as a means of root canal disinfection, compared with the traditional and less costly method of irrigating with sodium hypochlorite. However, it should be noted that the culture of E. faecalis was grown up overnight, and was predominantly a planktonic culture. As such, elimination of the bacteria was achieved much more easily, even with 1% sodium hypochlorite. The way in which the canal was irradiated was conducted with the fibre-optic tip placed in the apical and coronal aspects of the canal for two 15 second cycles in a “circumferential painting movement”. While the concept of activating an irrigant with laser energy had not been conceived at that time, the act of placing the laser tip within the root canal with the nigrosin dye in distilled water may have inadvertently activated it. It should also be noted that the Nd:YAG laser at 4.5W is at a fairly high power setting and may potentially result in apical extrusion of the irrigant in an in vivo situation.

As is the case in many in vitro studies, the bacteria culture is often an overnight culture. This is in contrast to the bacteria present in secondary or persistent root canal infection cases, such as E. faecalis, which have the propensity to invade dentinal tubules (Love, 2001). This would severely limit the ability of the photosensitiser dye to come into direct contact with the bacteria. Moreover, some of the studies that have examined the efficacy of photo-activated disinfection have utilised planktonic cultures of bacteria (Moshonov et al., 1995; Silbert, 2000). These results potentially may not be extrapolated to in vivo situations, where the bacteria, such as E. faecalis forms a biofilm, which provide increased protection against much of the anti-microbial agents in the form of irrigants and medicaments (Love, 2001).
1.9.2.2 Direct irradiation of root canals

Other investigators have examined the effect of directly irradiating the root canal with the laser on the eradication of bacteria. Much of this work has been conducted with the use of the Nd:YAG laser. Prior to the approval for its use in dentistry, Nd:YAG lasers had been used extensively in dermatology, ophthalmology, gynaecology and general surgery (Hardee et al., 1994). This laser was, at that time, arguably the most widely used laser in endodontics. It emits at a wavelength of 1,064 nm, which is in the near infra-red range (Rooney et al., 1994). Gutknecht et al. (1996) investigated the effect of this laser when irradiation was carried on the root canals of extracted teeth. The teeth consisted of both anterior and posterior teeth, and the root canals were infected with *E. faecalis* and cultured for a period of 24 hours. The results garnered for the experiment were impressive; when the canal was irradiated with the laser at 1.5W, a 99.91% reduction in bacteria was observed. However, it should be noted that the *E. faecalis* culture had been grown up for 24 hours only. As such, the bacteria might have only been a planktonic culture. The sampling technique employed in the study consisted of rinsing the canals of all the teeth with 1ml of 0.9% saline solution, and then collecting the eluate in a test tube. Serial dilutions were subsequently conducted using this eluate. This method may have missed some of the bacteria as the saline rinse may not have reached some parts of the canal, especially within the complex anatomy of multi-rooted posterior teeth, where isthmuses and joining canals exist.

A more recent study by Berkiten et al. (2000) also used a pulsed Nd:YAG laser and examined its effects on the disinfection of root canals infected with *Streptococcus sanguis* and *Prevotella intermedia* for 20 days. Given that the bacteria had been cultured within the root canals for 20 days, it would have been likely that the bacteria would have established a biofilm (Plutzer, 2009). Employing two power settings of 1.8W and 2.4W, they found that the former was able to eliminate 86.3% of *S. sanguis* with a mean penetration of 607.6 μm into dentinal tubules, while the higher power setting of 2.4W was capable of achieving 98.5% sterility. The laser at the setting of 2.4W was also capable of disinfecting up to a mean of 666.6 μm. The laser had also completely eliminated *P. intermedia* from the root canal at both settings (Berkiten et al., 2000). Results from light microscopy showed that a significant number of *S. sanguis* had penetrated the dentinal tubules. As such, this study provided evidence of the effectiveness of laser irradiation in the elimination of bacteria, and that the laser also had an effect on the bacteria that had penetrated dentinal tubules within the root canal. However, scanning electron microscopy employed in the study
revealed that the laser had caused alterations carbonisation of the dentinal walls, and closure of some dentinal tubules due to melting and recrystallisation of the radicular dentine. In addition, root canals lased at 2.4W also showed evidence of crumbling and fissures on the dentine layer (Berkiten et al., 2000). As such, the surface morphology of the radicular dentine had been altered irreversibly, and this may have ramifications on the penetration of root canal sealer into the dentinal tubules.

Another problem with the use of direct irradiation is the heat generated by the laser. A study by Hardee et al. (1994) to evaluate the effectiveness of Nd:YAG laser in sterilising root canals of single-rooted teeth infected with Bacillus stearothermophilus spores. These spores are extremely heat resistant, and were cultivated by incubating at 55ºC in the study. The canals which were irradiated for 1 min at 3W had a 98% reduction in colony forming units (CFU), while those that had been exposed to 3 minutes of 0.5% sodium hypochlorite registered an 88% reduction in CFU. However, the teeth group that had been irradiated for 1 minute became extremely hot and could not be handled by the end of the period of irradiation. The authors also noted charring of the dentine and production of smoke. In another 2 groups, laser irradiation was conducted for 1 and 2 minutes, with 0.5% sodium hypochlorite within the canal. This resulted in 98% and 99% reduction in CFU respectively, but the temperature of the teeth did not rise as rapidly. However, once the sodium hypochlorite had vaporised completely, these teeth also became too hot to touch.

In an in vivo situation, this would have adverse effects on the surrounding periodontium. In a histological study in dogs, Bahcall et al. (1992) reported periradicular osteocyte necrosis 24 hours after root canal debridement was performed with Nd:YAG laser irradiation for 30 seconds. These same teeth exhibited external resorption and ankylosis after 30 days, and it was postulated that the damage to the periradicular structures may have been the result of heat transfer from the tooth structure, or absorption of the laser light by the periradicular tissues. As such, it may be necessary to reduce either the time of irradiation, or the power output of the laser. However, this may compromise the effective disinfection of the root canal, because even at the settings used in the Hardee et al. (1994) study, complete sterilisation of the root canal was not achieved.

Ramsköld et al. (1997) sought to determine the thermal changes following irradiation with Nd:YAG laser in an in vitro setting. At 3W and irradiation for a period of 15 seconds, there was an increase of 6 ºC registered. As they irradiated the canal intermittently, there was a fall in temperature, followed by a subsequent increase during lasing of the canal. Temperatures as high as 42.6 ºC were recorded, while the lowest temperature reached as 36.7 ºC. It should be noted that the canals were dry during irradiation of the root canals. As
such, the amount of energy necessary to raise the temperature is lower, as compared to a canal which is wet. Water on the other hand, requires nearly four times the amount of energy as compared to dentine to raise the temperature to the same degree (Ramsköld et al., 1997). This is because the properties of water and dentine differ for both absorption and conduction of heat (Ramsköld et al., 1997). As such, a wet canal will subsequently be able to insulate against rapid increases in temperature. The higher density of dentine compared to water also translates into greater thermal diffusivity of dentine (Ramsköld et al., 1997). All these factors must be taken into consideration when deciding on the power output and timing for irradiating a canal during debridement, so as to prevent any potential damage to the periodontium.

More recently, other investigators have used other lasers, such as the erbium:yttrium-aluminum-garnet (Er:YAG) laser (Perin et al., 2004), and the erbium, chromium:yttrium-scandium-gallium-garnet (Er,Cr:YSGG) laser (Gordon et al., 2007; Schoop et al., 2007; Schoop et al., 2009), and some others have compared its effectiveness against other irrigation regimes in the disinfection of the root canal (Wang et al., 2007). These lasers emit at wavelengths of 2,940 nm and 2,780 nm respectively (Schoop et al., 2007). Their action is carried out through photoablation, as the wavelengths correlate closely with the absorption maximum of hydroxyapatite (Hibst et al., 1989). This has been found to be ideal for the removal of dental hard tissues, as water that is present within dentine evaporates instantaneously when irradiated, resulting in ablation of the tissue with minimal increases in temperature (Hibst et al., 1989). Due to this ability, much of the research conducted using these lasers have predominantly been on caries removal and cavity preparation (Hadley et al., 2000). However, in recent times, the effect of the Er,Cr:YSGG laser on the morphology of the root canal dentine following irradiation has been examined (Yamazaki et al., 2001; Altundasar et al., 2006). In one of the studies, irradiation with the Er,Cr:YSGG laser occurred at the power output of 3W, and scanning electron microscopy revealed several sites that had partial melting and occlusion of the dentinal tubules (Altundasar et al., 2006). Other areas showed carbonisation, despite the use of water coolant during irradiation (Altundasar et al., 2006).

Subsequent to these investigations, the use of the Er,Cr:YSGG laser in the disinfection of root canals was evaluated. Results from one of the studies showed that root canals infected with Escherichia coli and E. faecalis could not be completely sterilised (Schoop et al., 2007). This was despite the culture having been grown up within the root canals for 4 hours only, and was thus unlikely to have been a biofilm (Schoop et al., 2007). Using power output settings of 1W and 1.5W, increases of 2.7°C and 3.2 °C respectively
were recorded (Schoop et al., 2007). The authors believed that this range of increase was within safe limits. Scanning electron microscopy of the root canal dentinal walls showed minimal disruption to the natural topography, with patent dentinal tubules present after irradiation at 1W. Root canals that were irradiated with 1.5W, however, exhibited partial melting and recrystallisation of dentine, thereby occluding some of the dentinal tubules (Schoop et al., 2007).

Wang and co-workers (2007) set out to compare the bactericidal effect of irradiation of the root canal with the Er,Cr:YSGG and Nd:YAG lasers, against irrigation with 2.5% sodium hypochlorite. Using single rooted teeth with a single root canal, these teeth were infected with E. faecalis and allowed to incubate for 3 weeks prior to treatment. Irradiation of the root canals with the Er,Cr:YSGG and Nd:YAG lasers were at power outputs of 1W and 1.5W for a period of 40 seconds, and this would be compared against a 2 minute irrigation with 2.5% sodium hypochlorite. The Nd:YAG laser proved to be more effective than the Er,Cr:YSGG laser at reducing the number of CFU, as the former resulted in 97% (1W) and 98% (1.5W) reduction, while the latter was not as effective at the power setting of 1W, with 77% reduction in E. faecalis CFU. Irradiation with the Er,Cr:YSGG laser at 1.5W was more effective, resulting in 96% killing of the bacteria, but the traditional method of irrigation with sodium hypochlorite resulted in 100% reduction in bacteria. This study highlighted the effectiveness of sodium hypochlorite as an irrigant and why it has stood the test of time in the face of technological advancements in root canal debridement and disinfection methods. One of the strengths of the study was the cultivation of E. faecalis for 3 weeks, which meant that the different treatment modalities were working to eradicate a biofilm. It should be noted that sampling of the root canals occurred with paper points, and thus, there may still be E. faecalis present in the dentinal tubules or not detected with paper point sampling. As such, the 100% kill rate registered by sodium hypochlorite may not be entirely accurate, but this sampling method was standardised across all treatment groups.

Up to this time, all of the research with lasers had employed the use of end firing tips. The introduction of radial firing tips, which are conical in shape, was aimed at providing better coverage of the root canal (Gordon et al., 2007). Compared with the forward emission of laser energy that occurs with end firing tips, the radial firing tips were also thought to result in the irradiation of the dentinal tubules given the more favourable direction of laser emission, thereby potentially eliminating bacteria that penetrate the dentinal tubules (Gordon et al., 2007). This was tested with the use of dentine cylinders which were infected with E. faecalis (Gordon et al., 2007). Following the model described
by Haapasalo and Orstavik (1987), cementum from the root section were removed to facilitate invasion of the dentinal tubules. After incubating the bacteria for 1 week, the dentine cylinders were divided into different treatment groups which received either sodium hypochlorite irrigation or irradiation with the Er,Cr:YSGG laser at different power settings, timings, and with or without the water spray. The results showed that irradiation at 0.35W without water spray resulted in the greatest reduction in E. faecalis CFU. The authors believed that dry laser treatment meant that the laser had a direct effect on the water-containing bacteria, since the wavelength of the Er,Cr:YSGG laser is absorbed by the water (Gordon et al., 2007). When the water spray was used, the laser energy was diminished consequently. The penetration of the laser energy into dentine also contributed to the significant reduction in CFU, since the wavelength of the Er,Cr:YSGG is readily absorbed by dentine as a result of the hydroxyapatite and interstitial water present within. The pulse of this laser is composed of approximately 150 micropulses, and each micropulse causes penetration of energy of about 3 μm into interstitial water. As such, it is the opinion of the authors that expansion of intratubular water and collapse of the water vapour can occur up to 1000 μm into dentine, a phenomenon they termed “micropulse-induced sequential absorption”. This expansion and collapse was thought to be capable of generating acoustic waves which could disrupt bacteria that had penetrated into the dentinal tubules (Gordon et al., 2007).

The use of the radial firing tip was also evaluated by Schoop et al. (2009), assessing the bactericidal, morphological and thermal effects of the Er,Cr:YSGG laser in conjunction with the radial firing tip. Employing the laser at two power output settings of 0.6W and 0.9W, it was found that neither of these was capable of completely sterilising root canals that were infected with E. faecalis in their in vitro study. The advantage of using the laser at such low power settings meant minimal morphological alterations to the root dentine surface, and negligible increases in temperature (1.3 – 1.6 ºC) (Schoop et al., 2009). Given the results, it would seem that the effectiveness of the Er,Cr:YSGG laser in disinfection of the root canal is severely limited.

1.9.2.3 Laser activation of irrigants

When considering the use of the Er,Cr:YSGG laser in disinfection of the root canal system, a balance must be achieved between sufficient power output for effective sterilisation and avoidance of excessive morphological alterations to the root canal dentine. Due to the ablative nature of the Er,Cr:YSGG laser, the creation of ledges, especially
within curved canals, is a clear and present danger. The in vitro studies that have been carried have generally shown that at lower power outputs, complete sterilisation of the root canal with the Er,Cr:YSGG laser is generally unattainable (Gordon et al., 2007; Schoop et al., 2007; Schoop et al., 2009; Wang et al., 2007). It should be noted also that these studies were conducted on single rooted teeth with relatively simple anatomy. The ability of direct irradiation with the Er,Cr:YSGG to sterilise root canals with various anatomical variations, such as fins, anastomoses and isthmuses, is thus doubtful.

1.9.3 The mechanism underlying laser activation of irrigants - Cavitation

The immense of energy generated by laser irradiation could however be harnessed in a different manner. Similar to ultrasound, cavitation could potentially be generated by laser activation of a fluid, and the subsequent explosion and implosions could result in pressure waves (Blanken et al., 2009). These waves would subsequently create shear stresses along the dentinal walls of the root canal. The fluid has been shown to travel at up to 20 m/second (72 km/hour), and the speed has been attributed to vapour bubble expansion and implosion with secondary cavitation effects, thereby inducing such high speed fluid motions (Blanken and Verdaasdonk, 2007).

Using high speed imaging techniques and glass cylinders which simulate the root canal, images were captured and combined to show the dynamics of the generation and collapse of vapour bubbles in a time range from 1 to 300 – 3,000 microseconds (Blanken et al., 2009). When the laser pulse was fired, the energy was absorbed in a 2 mm-thick layer of fluid that was instantly heated to boiling temperature (100 ºC) at high pressure and converted into vapour. The high pressure of this vapour caused it to expand at high speed and provided an opening in front of the fibre for the laser light. At the subsequent pulse, the light emitted from the laser passed through the bubble and the water surface in front of the bubble is evaporated. Blanken et al. (2009) described it as the laser light having “drilled a channel through the liquid until the pulse ends after 140 microseconds”. This phenomenon has been described previously as the “Moses effect in the microsecond region” by van Leeuwen et al. (1991). On cessation of irradiation, cooling of the vapour occurred and condensation began. The momentum of the previous expansion, however, caused the creation of a region of lower pressure within the vapour bubble. Liquid that surrounded the bubble then accelerated to fill in the gap. Implosion of the vapour bubble then occurred near the tip of the fibre, and resulted in the separation of the bubble from the fibre. Fluid then rushed into the bubble, causing it to implode. The imploding bubble was
noticed to be shaped like a sickle. The process of implosion is completed after 260 microseconds, and the vapour bubble was no longer present. This is an occurrence which is reproducible in an open environment. However, within the confines of the root canal, the bubble cannot expand freely. Fluid is thus pushed forwards and backwards, leading to compression of the bubble into a flattened disk. The further need to increase in size is prevented by fluid that is apical to the expanding bubble, and thus, the bubble then grew in a coronal direction along the length of the laser fibre. The pressure within the bubble remained high for an extended period of time, given the resistance of fluid displacement within the space restrictions of the root canal. This thus delayed the dynamics of expansion and implosion, and it has been shown that it may take three times as long for cavitation to occur because lateral and forward expansion of the bubble would be prevented by the root canal wall, and backward expansion restricted by the fibre tip (Blanken and Verdaasdonk, 2007). However, the restricted space creates shear stresses along the wall of the canal, and potentially generates secondary cavitation bubbles as a result of the high speed fluid motion. The implosion of the primary and secondary bubbles creates microjets in the fluid aimed at the wall with very high forces locally (Blanken et al., 2009).

Other researchers have recorded similar findings utilising similar experimental set-ups (de Groot et al., 2009). They noted that a higher energy pulse would result in the longer growth time of the bubble, and that collapse of the bubble occurred with a velocity of 1 m/s. It was also noticed that when sodium hypochlorite was used as the irrigant, the bubble grew to a larger size, and consequently took a longer time before collapsing. A higher number of smaller bubbles were also present after laser activation with sodium hypochlorite as compared to water (de Groot et al., 2009). Hmud and co-workers (2010) examined the potential for two diode lasers, which operate at the wavelengths of 940 nm and 980 nm, to generate cavitation in a fluid medium. These smaller laser units are usually employed in soft tissue surgery, and the cost of each unit is generally less prohibitive compared with the solid state lasers. Because these lasers are within the near infrared spectrum, these wavelengths are “close to harmonics for water absorption”, and tend to be more strongly absorbed compared with other near infrared wavelengths of 810 nm, 830 nm and 1064 nm (Gutknecht et al., 2004). The results showed that the two laser systems were capable of inducing cavitation within a variety of fluid media, and that an increase in power output generally resulted in a decrease in the time taken to induce cavitation (Hmud et al., 2010). The presence of dissolved air within the fluid, however, failed to speed up the induction of cavitation, which leads to the conclusion that the bubbles that are formed within the fluid is indeed water vapour (Hmud et al., 2010).
1.9.4 Potential clinical applications of laser-induced cavitation

The potential of laser activation of irrigants has since been explored in the removal of smear layer. George et al. (2008) assessed the ability of two different lasers, the Er:YAG and the Er,Cr:YSGG, to activate different irrigants (water, hydrogen peroxide and EDTAC) and subsequent effectiveness at removing the smear layer from the apical third of root canals in an in vitro study. They also sought to determine if the two different tips, i.e. the end firing and the radial firing tips, would have any influence on the outcome. The results revealed that laser activation of the irrigants, in particular EDTAC, had a significant influence on removal of the smear layer. Water and hydrogen peroxide was found to be inferior to EDTAC in terms of smear layer removal. The authors did not find any difference between the two laser systems. However, the radial firing tips were shown to perform better than the end firing tips, when the same laser and same irrigant were used. The authors postulated that “hydraulic stresses created in the EDTAC solution by lasing might agitate it”, leading to an increase in its temperature and subsequent increase in penetration into the smear layer (George et al., 2008).

De Moor et al. (2009) have also evaluated the ability of laser activated irrigation in the removal of dentine debris in an in vitro model. They compared the efficacy of different modes of irrigation, namely, active hand irrigation, passive ultrasonic irrigation and laser activated irrigation. Using extracted single rooted teeth, the teeth were split and a groove made in the root canal to simulate uninstrumented canal extensions (De Moor et al., 2009). Dentine debris was placed into the grooves and the two halves were then reassembled. Active hand irrigation involved moving the needle up and down within the root canal, while ultrasonic activation of the irrigant was achieved with an Irrisafe tip (Satelec). The Er,Cr:YSGG laser was used to activate the irrigant at a setting of 75 mJ at 20 Hz. The irrigant used in the experiment was 2.5% sodium hypochlorite. The results showed that the laser activated irrigation was the most effective means, with 75% of canals having no debris left within the groove compared with 30% and 0% for ultrasonic activated irrigation and active hand irrigation respectively (De Moor et al., 2009).

As promising as the results reveal, there are considerations with regards to safety to the patient. A recent in vitro study by George and Walsh (2008) examined the extrusion of fluid through the apex following laser activation of irrigant with two types of lasers, the Er:YAG and Er,Cr:YSGG lasers, and two different types of tips, end-firing and radial firing tips. The lasers were set at a power setting of 4W and 1.25W respectively and placed at either 5 mm or 10 mm from the apex. This was compared against conventional hand
irrigation with two different types of needles, one a non-bevelled needle and the other a side venting needle. The teeth were mounted horizontally, the root canal was then filled with a dye solution and paper placed under the teeth samples to record the extruded dye. It was found that while neither laser type nor tip design were significant variables, the amount of apical extrusion from teeth that received laser activation of the dye was significantly higher than syringe irrigation (George and Walsh, 2008). There was no significant difference whether the laser tip was placed 5 mm or 10 mm from the apex, although teeth with the tip placed 5 mm from the apex had a greater amount of extrusion (George and Walsh, 2008). Results from the study clearly show that one of the risks of laser activated irrigation is apical extrusion of the irrigant and this may have deleterious effects on the periradicular tissues and to the patient. However, the authors did note that the in vitro study did not take the presence of an intact periodontium into consideration (George and Walsh, 2008). Nonetheless, the promising results as seen in the study by De Moor et al. (2009) must be tempered with caution, as the risk of irrigant extrusion must always be considered, especially if the irrigant used is sodium hypochlorite.

To date, most of the literature that has pertained to the use of the laser to eliminate infection from the root canal system through direct irradiation (Wang et al., 2007, Schoop et al., 2009), or to activate irrigants to examine the effects on removing dentinal debris (De Moor et al., 2009). As such, no study has examined the effects of laser energy induced cavitation on an antimicrobial irrigant to eliminate a bacterial biofilm.

1.10 Aim

At the moment, there is a paucity of studies that have examined the effectiveness of sonic or laser activation against a mature E. faecalis biofilm and the capacity to eradicate cells that have invaded the dentinal tubules. Given the highly promising results of many of the in vitro studies employing the use of the Er,Cr:YSGG laser activated irrigation in the removal of dentinal debris and the smear layer, it would of interest to see if the outcomes achieved by this device could translate into more efficacious eradication of bacteria. Also, to date, there are no studies that have investigated the effectiveness of single visit retreatment against a multiple visit protocol. Perhaps the power of laser activated irrigation may provide the predictability in bacterial eradication that endodontics has been seeking, and thus allow single visit retreatment to be a much more viable option. Moreover, this study also hopes aid clinicians in comparing between a simple device that is relatively inexpensive and compact, with one that is much more expensive and elaborate.
The aim is to compare activation of irrigants achieved through acoustic streaming via the EndoActivator® sonic device, and cavitation resulting from the Er,Cr:YSGG laser against an *E. faecalis* biofilm cultivated within root canals of extracted anterior teeth.
Chapter 2. Materials and Methods

2.1 Flow cell

The study employed the use of a continuous flow cell which was designed to establish a biofilm within decoronated tooth roots. The body of the flow cell was constructed from Delrin poly-acetyl resin (Dupont, Wilmington, DE, USA), while the viewing window of the flow cell and the plate on which tooth roots were mounted was made from Perspex. The flow cell dimensions were 200 mm (W) × 200 mm (L) × 60 mm (H). A growth medium inlet and outlet were secured to the top Perspex plate, while another outlet was situated on the lower half of the flow cell. The inlet allowed the flow of growth medium into the flow cell, while the upper outlet allowed air to escape (Figure 4).

![Figure 4. Diagram of flow cell](image)

The flow cell is divided into two chambers, separated by the Perspex plate on which the tooth roots are mounted. Holes were drilled into this Perspex plate to accommodate 20 tooth roots, and the plate was secured onto the body of the flow cell with stainless steel screws. The depth of the upper chamber was approximately 4.0 mm and acted as a reservoir for growth medium flowing into the flow cell. The growth medium flowed into the lower chamber through the root canals of the tooth roots. The second outlet located in the lower chamber allowed the growth medium to be removed from the flow cell. Two neoprene gaskets were used to ensure that the flow cell only allowed passage of the growth medium through the tooth root canals.
2.2 The test organism

The *Enterococcus faecalis* (V583, ATCC 700802) was purchased from Cryosite (New South Wales, Australia). The organism was maintained on Todd Hewitt Broth agar (Oxoid, Victoria, Australia), and the purity of the cultures was periodically checked by Gram Staining. Frozen stock cultures were kept in 40% v/v glycerol at -80 ºC.

For flow cell inoculation, the organism was cultured overnight in 50 ml Todd Hewitt Broth (Oxoid, Victoria, Australia).

2.3 Preparation of teeth

Teeth used in this study were obtained from the Oral Surgery Department of the Adelaide Dental Hospital. Ethics approval had been obtained for the use of de-identified, extracted teeth from the University of Adelaide Human Research Ethics Committee.

Teeth selected for the study were single rooted teeth with completed apices. They were stored in 0.02% thymol solution at 25 ºC until ready for use. The teeth were closely examined for caries and resorptive lesions and teeth that had extensive root caries, where the cementum was deemed to have been severely compromised, were discarded. All remnants of soft tissue were removed mechanically, and calculus was removed with an ultrasonic scaler (EMS, Nyon, Switzerland) set at a power setting of 70%, prior to root canal preparation.

The teeth were decoronated with the use of a diamond coated disc (3M, Minnesota, USA) in a laminar flow hood, and the length of each root was then adjusted to 15.0 mm. A glide path was first established with the use of size #10 and #15 stainless steel K-files (SybronEndo, California, USA). Mechanical instrumentation of the root canal was subsequently performed with K3 rotary NiTi files (SybronEndo, California, USA). The canals were prepared with file sizes #25/0.10, #25/0.08, #25/0.06, #35/0.04, #35/0.06 and #40/0.06, using a crown down technique. Final instrumentation with the #40/0.06 was done so that the file would penetrate 1.0 mm beyond the apex of the root. The rationale for this would be to facilitate the flow of media through the tooth root for establishment of a biofilm within the root canal.

During mechanical preparation, a total of 20.0 ml of 17% ethylenediamine tetra-acetic acid (EDTA) was used to irrigate the root canal. EDTA was left within the root canal for 1 minute prior to completion of debridement, so as to ensure thorough removal of the smear layer produced during instrumentation. Irrigation was performed with a 3.0 ml
syringe with a 27-gauge needle. Paper points were used to dry the canals, and the external surfaces of the teeth were then allowed to air dry. Handling of the teeth was facilitated by placing two size #40 paper points into the root canal, and two coats of nail varnish were applied over the entire root surface, as well as the coronal aspect. One of the paper points was pushed through the apex so as to prevent the nail varnish from occluding the apex of the tooth root. The presence of the paper points also prevented nail varnish from covering the orifice of the root canal, or flowing into the root canal.

When the nail varnish had dried, the paper points were removed and the roots were mounted onto the Perspex plate by syringing medium body polyvinyl siloxane impression material (3M ESPE, Seefeld, Germany) around the root, up to 5.0 mm in thickness, which was the thickness of the Perspex plate. The roots were mounted such that the coronal aspect of the tooth root and the orifice of the root canal were flushed with the superior aspect of Perspex plate, while the rest of the root would be projecting through the inferior aspect of the Perspex plate (see Figure 1). The Perspex plate containing the 20 root samples was then secured onto the flow cell with stainless steel screws.

A total of 100 teeth were initially planned for use in the treatment groups. However, there was a scarcity of supply of single canal, single rooted teeth and six of those teeth were deemed to have root caries that was too extensive for consideration in the study. 92 teeth were used. Four of these teeth were used in a pilot study to determine if an \textit{E. faecalis} biofilm could be established in the root canals. These four teeth were analysed using scanning electron microscope (SEM) imaging to visualise the biofilm.

\subsection*{2.4 Sterilisation of the flow cell}

The flow cell was set up with the tooth roots in place, then closed and secured with screws. After attaching the silicone and peristaltic pump tubing, the entire apparatus was placed into a sterilisation bag. The apparatus was sterilised by gamma irradiation at 2 kGy (Steritech Pty Ltd, Dandedong, Victoria, Australia). This method is an extremely effective sterilisation method and has been shown to cause minimal disturbances to the dentine (White \textit{et al}., 1994).

\subsection*{2.5 The flow cell apparatus}

The continuous flow system consisted of a nutrient reservoir, the flow cell, a peristaltic pump, and a waste vessel (Figure 5). The nutrient reservoir, containing Todd
Hewitt Broth (Oxoid, Victoria, Australia) and waste vessel were sterilised by autoclaving at 121 ºC for 20 min and the system was then attached aseptically to the flow cell. The flow cell was placed on a heating bed, and the temperature maintained at 37 ºC.

**Figure 5.** Flow cell apparatus consisting of a nutrient reservoir (Todd Hewitt Broth) pumped into the flow cell via the peristaltic pump and out into the waste receptacle.

Todd Hewitt Broth (THB) (Oxoid, Victoria, Australia) was pumped into the flow cell using a peristaltic pump. When the flow cell was filled the peristaltic pump was stopped and air bubbles were cleared from the flow cell through the upper outlet. 50 ml of *E. faecalis* culture grown overnight in THB (Oxoid, Aust.) was introduced into the flow cell through a syringe injection port located upstream of the flow cell. After 24 hrs, laminar flow was established at a rate of speed of 23 ml/hr, giving a dilution rate of 0.038 hr⁻¹.

The continuous flow cell was allowed to run for 4 weeks, as a previous pilot study using 4 tooth root samples in our laboratory had shown that growth over 4 weeks produced a confluent biofilm over dentine disks.

### 2.6 Sampling of root canals

At the end of four weeks, the silicone tubes connected to the flow cell via the inlet and two outlets were clamped with artery forceps and cut to isolate the flow cell. The flow cell was opened and the THB (Oxoid, Victoria, Australia) removed. Using sterile,
autoclaved instruments Cavit (3M ESPE, Seefeld, Germany) was then placed to seal the orifice and the apex of the roots. Two coats of nail varnish were painted over the Cavit (3M ESPE, Seefeld, Germany) and a hair dryer was used to hasten the drying process.

When the nail varnish had set, the flow cell was then filled with 4% sodium hypochlorite (Endosure, Hypochlor, Dentalife, Croydon, Australia) and allowed to soak for 1 hour. This was to eliminate any bacteria that may be on the root surfaces, as it has previously been shown that exposure of an *E. faecalis* biofilm to 4% sodium hypochlorite for 60 minutes was sufficient to eliminate 99.99% of the bacteria (Plutzer et al, 2009). Placing Cavit (3M ESPE, Seefeld, Germany) and nail varnish over the orifices and apices prevented penetration of Sodium hypochlorite into the root canal. After 1 hour, the 4% sodium hypochlorite was removed and the flow cell rinsed with 5% sodium thiosulphate to neutralise the effects of sodium hypochlorite and prevent any residual anti-microbial action from influencing the results.

Cavit (3M ESPE, Seefeld, Germany) and nail varnish from the orifice was then removed with a sterile instrument. The Cavit (3M ESPE, Seefeld, Germany) and nail varnish placed at the apices were left intact, simulating the periapical tissues *in vivo*. Irrigation of the root canal by syringe or activation with a sonic instrument or laser, was then performed according to the allocation of the tooth root to the respective treatment group. There were a total of 6 treatment groups. During irrigation and due to the proximity of the teeth, sterile gauze was placed around the orifice of the root canal to prevent the irrigant from flowing onto adjacent teeth. This additional measure was taken despite the presence of Cavit (3M ESPE, Seefeld, Germany) and nail varnish occluding the orifices of the adjacent teeth.

When irrigation or activated irrigation was complete, two size #40 paper points were used to soak up the canal contents. These were then streaked onto a Todd Hewitt agar plate to detect growth of *E. faecalis*. One tooth from each group was always selected randomly for scanning electron microscopy (SEM) and immersed in fixative (4% paraformaldehyde/1.25% glutaraldehyde in phosphate buffered saline (PBS) + 4% sucrose) for at least 24 hours. All other tooth root samples were removed from their mounting on the Perspex plate with sterile forceps and placed into a sterile custom made tooth-crusher. This device was constructed from brass, and consisted of a piston which fitted closely into a cylinder, into which the root was placed (Figures 6 to 8). The piston was inserted into the cylinder and a hammer used to hit the piston. On removal of the piston, 2 ml of 0.9% sterile saline was added to the cylinder containing the crushed tooth fragments. The contents were then poured out into a sterile tube and labelled. Between samples, the piston
and cylinder were rinsed with 10 ml of 70% ethanol to eliminate bacteria from the previous sample, and the effect of the ethanol was then neutralised by a subsequent rinse with 40 ml 0.9% sterile saline.

Figure 6. Instrument for crushing teeth consisting of a brass piston (left) and cylinder (right).

Figure 7. Brass container with tooth sample placed within.
2.7 Determination of cellular viability and protein concentration

The sterile tubes containing the crushed tooth root samples in 0.9% sterile saline were then vortexed for 30 seconds. Serial dilutions from $10^{-1}$ to $10^{-4}$ were made for each sample in 0.9% sterile saline, and 100 μl aliquots of each dilution were plated in duplicate onto THB agar (Oxoid, Victoria, Australia) plates. The plates were then incubated aerobically at 37°C for 24 hours, and the number of colony forming units (CFU) per ml were counted.

Cellular protein levels of the crushed root suspension were then determined to allow normalisation of the amount of harvested bacterial biofilm within the root canal. For the protein assay, 100 μl of 1M sodium hydroxide (NaOH) was added to 900 μl of suspension and placed in a boiling water bath for 30 minutes. 150 μl of each sample was then pipetted into microtitre plate wells in triplicate before the addition of 150 μl of Coomassie Plus protein assay reagent (Pierce biotechnology, Rockford, Illinois, USA). The microtitre plate was then shaken for 5 minutes, and the absorbance read at 595 nm using the microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA). The concentration of the samples
were standardised against known dilutions of bovine serum albumin (BSA), which were assayed in parallel with the samples. The CFU/ml/mg of protein was then calculated by dividing the CFU/ml by the cellular protein levels per ml of suspension.

2.8 Treatment groups

A total of 58 teeth were subject to 6 different irrigation protocols.

- **Saline** \((n=6)\)
  - Root canals were irrigated with 5 ml of 0.9% sterile saline, for a period of 60 seconds with a syringe and 27-gauge needle. The tip of the needle was placed within 2 mm of the apex.

- **Saline + sonic activation** \((n=11)\)
  - Using a 27-gauge needle, the root canals were irrigated with 5 ml of 0.9% sterile saline for 5 seconds, followed by 10 seconds of sonic activation with the EndoActivator (Dentsply, Maillefer, Ballaigues, Switzerland). This cycle was repeated 4 times, for a total of 60 seconds.
  - The size of polymer tip used in the EndoActivator was in accordance with the manufacturer’s recommendation which was to select the tip which would be accommodated comfortably within 2 mm of the apex. As such, a #30/0.04 tip was selected.
  - The EndoActivator was then set at its maximum power setting, which was 10,000 cycles per minute, which equates to about 166 Hz.

- **Saline + laser activation** \((n=12)\)
  - Using a 27-gauge needle, the root canals were irrigated with 5 ml of 0.9% sterile saline for 10 seconds, followed by 5 seconds of laser activation with the erbium, chromium:yttrium-scandium-gallium garnet (Er,Cr:YSGG) laser (Waterlase, Biolase Technology, San Clemente, USA). This cycle was also repeated for 4 times, for a total of 60 seconds.
  - The tip used was a radial firing tip \((17 \text{ mm}, 52^\circ)\), and 4 mm of the firing tip was inserted into the canal. The tip was withdrawn coronally during activation of the irrigant.
  - Laser activation of the irrigant was performed at a power output of 0.25W, with a pulse rate of 20 Hz, in the presence of air spray set at 10%. No water spray was activated in this study.
Sodium hypochlorite \( (n=7) \)
- The root canals were irrigated with 5 ml of 4% sodium hypochlorite for a period of 60 seconds with a syringe and 27-gauge needle. The tip of the needle was placed within 2 mm of the apex.
- At the end of the 60 second period, the sodium hypochlorite was neutralised with 5ml of 5% sodium thiosulphate for 60 seconds to prevent any residual anti-microbial activity of sodium hypochlorite.

Sodium hypochlorite + sonic activation \( (n=11) \)
- This treatment protocol was the same as that described for saline + sonic activation, except that 4% sodium hypochlorite was used instead of saline. Again, after the irrigation protocol was complete, 5 ml of 5% sodium thiosulphate was used to neutralise the effect of sodium hypochlorite.

Sodium hypochlorite + laser activation \( (n=11) \)
- The sequence of irrigation protocol was the same to that described for saline + laser activation, except that 4% sodium hypochlorite was used instead of saline, and at the end of treatment, 5 ml of 5% sodium thiosulphate was employed to neutralise any residual anti-microbial effect of sodium hypochlorite.

### 2.9 Scanning electron microscopy (SEM)

Following storage in fixative for a period of at least 24 hours, the roots were split into 2 halves for scanning electron microscopy (SEM). A cotton pellet was first placed in the orifice of the root canal to prevent any dentine debris from entering the root canal. The root surface was then notched with a diamond coated disc, and then the tooth root was split with a sterile plastic instrument. The entire procedure was conducted in a laminar flow hood.

Processing of the samples occurred in the following manner:
- Wash in PBS + 4% sucrose
- Dehydration of the samples in ascending concentrations of ethanol
  - 70% ethanol – 15 minutes
  - 90% ethanol – 15 minutes
  - 95% ethanol – 15 minutes
  - 100 % ethanol – 3 changes of 10 minutes each
After the samples had undergone critical point of drying, they were coated with carbon and gold and analysed under a scanning electron microscope (Philips XL 30, field emission scanning electron microscope). The confluence of the bacterial biofilm, the density of bacterial cells and the penetration into dentinal tubules were all assessed and images captured. A total of 30 root samples were used for imaging.
Chapter 3. Results

3.1 Biofilm growth

Preliminary experiments were conducted to investigate the optimum time for a confluent biofilm to be established within the root canal system. A previous study by Plutzer (2009) had found that a 4 week old biofilm consisted of “dense, multi-layered, confluent aggregations” of bacteria. However, the study had used dentine discs, and as such, it was crucial that the time frame be established to ensure that a sufficiently mature biofilm could be established within the in vitro root canal system.

At the end of the 4 weeks, tooth root samples were examined under scanning electron microscopy. It was found that dense aggregates of bacteria were adherent to the dentinal walls of the root canal, (Figure 9). Between the bacterial aggregates were strands of extracellular, amorphous material (Figure 10).

Figure 9. SEM image of 4 week old biofilm (6500× magnification)
Figure 10. SEM image of 4 week old biofilm (12,000× magnification)

<table>
<thead>
<tr>
<th>Percentage kill *</th>
<th>Experiment 1 n=10</th>
<th>Experiment 2 n=12</th>
<th>Experiment 3 n=14</th>
<th>Experiment 4 n=12</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Saline + sonic</strong></td>
<td>54%</td>
<td>96%</td>
<td>90%</td>
<td>80%</td>
<td>80%</td>
<td>23%</td>
</tr>
<tr>
<td><strong>Saline + laser</strong></td>
<td>49%</td>
<td>61%</td>
<td>87%</td>
<td>94%</td>
<td>73%</td>
<td>21%</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>10%</td>
<td>88%</td>
<td>50%</td>
<td>80%</td>
<td>57%</td>
<td>35%</td>
</tr>
<tr>
<td><strong>NaOCl + sonic</strong></td>
<td>95%</td>
<td>99.84%</td>
<td>66%</td>
<td>99.71%</td>
<td>90%</td>
<td>16%</td>
</tr>
<tr>
<td><strong>NaOCl + laser</strong></td>
<td>99.99%</td>
<td>99.99%</td>
<td>99.71%</td>
<td>99.99%</td>
<td>99.93%</td>
<td>0.14%</td>
</tr>
</tbody>
</table>

Table 1. Effect on viability of *E. fae calis* grown as a biofilm

* the percentage kill (%) was calculated using the equation

\[
\text{Percentage kill} = \frac{(\text{Control cfu/ml(saline)} - \text{treatment group cfu/ml}) \times 100}{\text{Control cfu/ml}}
\]

Although protein assays had been conducted to determine the CFU/μg of protein, the amount was too small to be detected in many of the samples and as such, the results were expressed as CFU/ml.
3.2 Treatment groups

3.2.1 Syringe irrigation with saline

Canals that had been syringe irrigated with saline had a significant bacterial load, and this was reflected in the SEM images, which showed almost no alteration or reduction in the dense biofilm (Figure 11). The remaining bacteria exhibited the architecture of the biofilm which had matured over 4 weeks, with the multiple layers of *E. faecalis* and strands of amorphous material laid down amongst the co aggregated bacteria (Figure 12). The openings of the dentinal tubules were also congested with *E. faecalis* cells. As such, syringe irrigation with saline was the positive control against which the other treatment regimens were compared to calculate the percentage kill of *E. faecalis* (Table 1).

![SEM image of dentine surface following syringe irrigation with saline (10,000× magnification)](image)
3.2.2 Syringe irrigation with sodium hypochlorite

Generally, the root canals that had been irrigated with sodium hypochlorite showed a decrease in the number of *E. faecalis* cells compared to those that had been irrigated with saline, given that it was able to achieve a mean percentage kill of 57% ± 35% (Table 1).

Evidence of surface bacterial killing was clearly present on the SEM images, as exposure of the biofilm to sodium hypochlorite caused the removal of much of the bacterial biofilm (Figures 13 and 14). The confluent, multi-layered architecture was no longer present, and only pockets of cell aggregates remained. In some areas of the radicular dentine, only single or pairs of cells were present (Figure 13).
Some cells were visible at the entrance of the dentinal tubules (Figure 15), but it was clear that a significant number of *E. faecalis* cells had invaded the dentinal tubules (Figures 16 to 18).
Figure 15. SEM image of *E. faecalis* at the entrance of the dentinal tubule following syringe irrigation with 4% sodium hypochlorite (15,000x magnification)

Figure 16. SEM image of *E. faecalis* within the dentinal tubules following syringe irrigation with 4% sodium hypochlorite (10,000x magnification)
**Figure 17.** SEM image of *E. faecalis* within the dentinal tubules following syringe irrigation with 4% sodium hypochlorite (20,000x magnification)

**Figure 18.** SEM image of *E. faecalis* within the dentinal tubules following syringe irrigation with 4% sodium hypochlorite (35,000x magnification)
3.2.3 Sonic activation of saline

The activation of the irrigants also resulted in a decrease in the CFU/ml. Sonic activation of saline when averaged over 3 experiments (n=2 in experiments 1, 2, and 4; n=3 in experiment 3) gave a percentage kill of 80% ± 23% (Table 1). While the numbers do reflect a trend in the killing rate following the use of the EndoActivator compared to syringe irrigation, the decline in the number of bacteria is not distinguishable on the SEM images (Figures 19 and 20). The dentinal wall still showed a dense biofilm, and it was evident that some cells were at the entrance of the dentinal tubules (Figure 20), and possibly had invaded the dentinal tubules. However, it can be seen that despite the extensive covering of the radicular dentine, the layer of biofilm is not as thick in some areas as compared with the biofilm on the root canals that had been irrigated with saline only (Figure 11).

![Figure 19. SEM image of E. faecalis on the root canal dentine surface following sonic activation of saline (6,500× magnification)](image-url)
3.2.4 Sonic activation of sodium hypochlorite

The average (n=2 in experiments 1, 2 and 4; n=3 in experiment 3) percentage kill for sonic activation of sodium hypochlorite with the EndoActivator was 90% ± 16% (Table 1). This was evident in the SEM images of the root canal that had received sonic activation of sodium hypochlorite, which showed relatively clean dentinal walls (Figure 21). Some areas of the radicular dentine were almost completely devoid of cells, except for cells occurring singly or in pairs (Figures 22 and 23), while other aspects of the root canal still harboured *E. faecalis* in small clusters (Figure 21). Again, there were signs of bacterial cells at the entrances of the dentinal tubules (Figures 23 and 24).
Figure 21. SEM image of *E. faecalis* on the dentine surface following sonic activation of 4% sodium hypochlorite (2,000× magnification)

Figure 22. SEM image of *E. faecalis* on the dentine surface following sonic activation of 4% sodium hypochlorite (6,500× magnification)
3.2.5 Laser activation of saline

The average (n=2 in experiments 1 and 4; n=3 in experiments 2 and 3) percentage kill for laser activation was 73% ± 21% (table 1). The use of the Er,Cr:YSGG laser to
activate saline resulted in no visible damage to the dentinal walls that could be discerned on the SEM images although it was evident that the biofilm has been disrupted. Exposed areas of the root canal dentine can be seen, but areas of dense aggregates of *E. faecalis* were present (Figure 25). The fibrillar, extracellular matrix is still evident, but the overall architecture of the mature four-week old biofilm has been destroyed (Figure 26).

However, it is evident from the dentinal surface that the dentinal tubules harboured *E. faecalis* (Figure 27). Imaging of the longitudinal sections of the dentinal tubules revealed dense aggregates of *E. faecalis* cells in some of the tubules (Figures 28 to 30). They were arranged mainly in pairs and short chains, although some of the tubules had single cells. Clearly, during the formation and maturation of the biofilm, some of the *E. faecalis* cells had also invaded the dentinal tubules.

![SEM image of E. faecalis on the root canal dentine surface following laser activation of saline (3,500× magnification)](image)

**Figure 25.** SEM image of *E. faecalis* on the root canal dentine surface following laser activation of saline (3,500× magnification)
Figure 26. SEM image of *E. faecalis* on the dentine surface following laser activation of saline (8,000× magnification)

Figure 27. SEM image of *E. faecalis* at the entrances of dentinal tubules following laser activation of saline (12,000× magnification)
**Figure 28.** SEM image of *E. faecalis* within the dentinal tubules following laser activation of saline (8,000× magnification)

**Figure 29.** SEM image of *E. faecalis* within the dentinal tubules following laser activation of saline (12,000× magnification)
Figure 30. SEM image of *E. faecalis* within the dentinal tubules following laser activation of saline (25,000× magnification)

3.2.6 Laser activation of sodium hypochlorite

The effectiveness of laser activation of sodium hypochlorite was very consistent across the different flow cells, with a mean percentage kill of 99.93% ± 0.14% (n=2 in experiments 1, 2 and 4; n=3 in experiment 3).

Clean dentinal walls that were devoid of bacteria were commonly seen in SEM images of the root canals that had laser activation of sodium hypochlorite (Figure 31). The dentinal tubules were not occluded by the biofilm, and rarely were there any signs of *E. faecalis* cells at the entrances of the dentinal tubules (Figure 32). Imaging of the longitudinal sections of the dentinal tubules also revealed no evidence of bacteria within the tubules adjacent to the root canal (Figures 33 to 35).
Figure 31. SEM image of the dentine surface following laser activation of 4% sodium hypochlorite (2,500× magnification)

Figure 32. SEM image of the dentine surface following laser activation of 4% sodium hypochlorite (8,000× magnification)
Figure 33. SEM image of the dentinal tubules following laser activation of saline (12,000× magnification)

Figure 34. SEM image of the dentinal tubules following laser activation of saline (20,000× magnification)
Figure 35. SEM image of the dentinal tubules following laser activation of saline (50,000× magnification)
Chapter 4. Discussion

The aim of the present study was to compare the effectiveness of sonic and laser activation of sodium hypochlorite, against traditional syringe irrigation, in the eradication of an *Enterococcus faecalis* biofilm. There are a number of studies that have shown the effectiveness of various antimicrobial agents against bacteria. However, much of the work has been conducted with the use of planktonic cultures (Schoop et al., 2007) which do not accurately reflect the manner in which bacteria grow within the root canal system. Nair (1987) had shown, with the aid of light and electron microscopy, that bacteria grow as a biofilm within the radicular dentinal walls, although the term „biofilm” was not used at that time. Since then, in–vitro studies have attempted to replicate in-vivo conditions by cultivating bacteria as a biofilm (Dunavant et al., 2006; Gutknecht et al., 1996; Spratt et al., 2001). However, the „biofilm” had been developed over periods ranging between 24 to 48 hours. Other studies have cultivated biofilms for longer periods of time, e.g. up to 3 weeks (Berber et al., 2006). In the present study, a biofilm model has been designed which is based loosely on the flow cell first described in the Dunavant et al. (2006). However, instead of using ceramic coupons (Dunavant et al., 2006) or dentine discs (Plutzer, 2009), root canals of extracted single canal teeth were used so as to recreate a more clinically relevant environment for growth of the biofilm. The use of ceramic coupons or dentine discs allowed the various antimicrobial agents to have maximum contact against the bacterial biofilm. Bacteria often invade the dentinal tubules (Love and Jenkinson, 2002), which limits exposure to antimicrobials and this could not be easily replicated with the use of flat discs.

The selection of *E. faecalis* as the bacteria for the present study was due to its frequent association with persistent endodontic infections (Molander et al., 1998; Sundqvist et al., 1998; Peciuliene et al., 2000; Pinheiro et al., 2003). In many of these studies, it was found that not only was *E. faecalis* the most commonly recovered microorganism from root canals of teeth with persistent endodontic disease, it was also often present within the canal as a mono-infection (Sundqvist et al., 1998; Peciulene et al., 2000; Pinheiro et al., 2003b). The results of these studies clearly demonstrate the dominance of *E. faecalis* in retreatment cases. The significant proportion of root canals of teeth exhibiting signs of persistent infection from which *E. faecalis* is recovered as a pure culture justifies the use of this microorganism for this study. The high incidence of recovery of *E. faecalis* from root canals of failed endodontic treatment also reflects the tenaciousness of the organism (Love, 2001; Evans et al, 2002) and highlights the importance of seeking an efficacious and
effective means to eradicate it, so as to eliminate disease and facilitate healing of the periapical tissues.

The choice of 4% sodium hypochlorite for the present study was due to its widespread use in endodontic practice, its broad spectrum of antimicrobial action and its ability to hydrolyse necrotic pulp tissue (Pashley et al., 1985; Zehnder, 2006). Due to short period of time for irrigation (1 min) and the small volume of irrigant employed in this study, it was deemed that a concentration of sodium hypochlorite that was sufficiently high to exert its antimicrobial effect was necessary. Moreover, no instrumentation was performed in this study, so the eradication of the *E. faecalis* biofilm was dependent solely on the antimicrobial effect of sodium hypochlorite.

This study is the first to investigate the effects of Er,Cr:YSGG laser activation of an antimicrobial irrigant against a bacterial biofilm. Also, to our knowledge, no *in-vitro* studies have been conducted whereby a bacterial biofilm has been cultivated within the root canal of extracted teeth. Little is also known of the effect of activating sodium hypochlorite solution with the Er,Cr:YSGG laser against the *E. faecalis* biofilm. Compared to previous studies, which investigated the use of the laser activated irrigation in the removal of dentinal debris and smear layer (George et al., 2008; de Groot et al. 2009; de Moor et al., 2009), the present study sought to determine the effectiveness of the Er,Cr:YSGG laser activation of sodium hypochlorite in the removal of a mature four-week old *E. faecalis* biofilm. Most studies that had examined the use of the Er,Cr:YSGG laser in the eradication of *E. faecalis* employed the laser without an irrigant present within the canal and directly irradiated the root canal (Gordon et al. 2007; Schoop et al., 2007; Wang et al., 2007). With the exception of the Wang et al. (2007) study, most of these investigations had cultivated *E. faecalis* for relatively short periods, ranging from 4 hours to 7 days (Schoop et al., 2007; Gordon et al., 2007). Based on a previous investigation, cultivation of the *E. faecalis* biofilm for a period of 4 weeks yielded a dense, multi-layered aggregation of cells within an amorphous extracellular matrix (Plutzer, 2009). It is evident that in our model that a 4 week period permitted the growth of a dense, mature biofilm, with cells that clearly had penetrated the dentinal tubules. When studying the effectiveness of an irrigation protocol, there are vast differences between the conditions of *in vivo* and *in vitro* studies. In some *in vitro* studies, a high volume of the irrigant is often available to directly contact bacteria which are often grown as a planktonic culture, resulting in optimal conditions for the eradication of the microorganisms (Gomes et al., 2001; Vianna et al., 2004; Dunavant et al., 2006). In the present study, although there was an attempt to
simulate in vivo conditions as much as possible, it is acknowledged that nutrient supply, redox and pH may not truly reflect the “in vivo lifestyle”.

The power setting of lasers in different in-vitro studies have ranged from 1.25W to 1.5W (George and Walsh, 2008; De Moor et al., 2009). However, these settings were deemed to be too high for use within the root canal, especially in conjunction with sodium hypochlorite. Preliminary visual investigations showed that the Er,Cr:YSGG laser at these settings caused a significant amount of agitation when the tip was placed in a beaker of water. Placing the laser tip into a plastic block simulating the root canal also caused a significant amount of extrusion through the „apex“ of the block at power settings of 1W to 1.5W. The lowest power setting on the Waterlase unit was 0.25W, and it was found that when only 4 mm of the laser tip was immersed in the irrigant, the amount of extrusion was minimised.

The selection of the EndoActivator® sonic instrument for comparison stemmed from its recent release, and it was our intention to test these novel devices to activate irrigants. The EndoActivator® comprises of a handpiece with detachable, single-use polymer tips of various sizes. Its ease of use, coupled with its cordless, lightweight design and relatively low cost, makes it a viable option for the endodontic practice. This provided an interesting contrast against the solid-state Er,Cr:YSGG laser which is significantly more expensive. The use of these two instruments permitted examination and comparison of the effectiveness of laser energy induced cavitation against the acoustic streaming achieved by the sonic activation of the irrigant.

4.1 Comparing syringe irrigation with 0.9% saline and 4% sodium hypochlorite

The results of this study illustrate clearly that the use of an antimicrobial irrigant such as sodium hypochlorite provides more effective eradication of bacteria as compared to saline. Using scanning electron microscopy (SEM), canals that had saline irrigation with a syringe showed minimal disruption of the E. faecalis biofilm. Syringe irrigation with 4% sodium hypochlorite was capable of achieving a percentage kill of 57% ± 35%. This finding was confirmed with the scanning electron microscopy (SEM) images. Following syringe irrigation with saline, the E. faecalis biofilm was thick and composed of multiple layers (Figure 11) and had barely been disrupted (Figure 12). The bacterial cells could be seen occluding the dentinal tubules (Figure 12), and was similar in appearance to the 4 week old biofilm which had been cultivated in our pilot study to evaluate the optimal time
for the biofilm development (Figures 9 and 10). SEM images of the radicular dentinal wall from samples that received sodium hypochlorite as an irrigant applied with a syringe showed that irrigant had eradicated most of the bacteria, leaving small groups or pairs of bacterial cells (Figures 13 and 15). Much of the canal wall appeared to be free from E. faecalis, although many had invaded the dentinal tubules, and had not been completely eradicated following irrigation with sodium hypochlorite (Figures 16 to 18). Under lower magnification, it is evident that most of the dentinal tubules contained E. faecalis (Figure 16).

The results are consistent with the findings of Byström and Sundqvist (1983), whose results showed that in 8 out of 15 canals, bacteria could not be recovered with paper points sampling after irrigation with saline. This was compared to 12 out of 15 canals that were irrigated with 0.5% sodium hypochlorite. However, unlike the study by Byström and Sundqvist (1983) which examined the efficacy of mechanical instrumentation combined with an antimicrobial irrigant, the present study only investigated the effects of irrigation. Despite the consistency of the findings with available literature, the percentage kill following syringe irrigation with sodium hypochlorite was somewhat less consistent. The percentage kill ranged from 10% (Experiment 1) to 88% (Experiment 2). One of the reasons for this may be due to the variability of the root canals. Despite attempts to standardise the root canal lengths (15 mm) and preparation of the root canal (#40, 0.06), the volume of the root canal was not equal in all teeth samples, and may have had an effect on the percentage kill. Increasing the volume of irrigant and placement of the needle to approximately 1 mm from the apex has been shown to significantly increase the effectiveness of bacterial elimination (Sedgley et al., 2005b). Similar to our study, no mechanical instrumentation was conducted in the study by Sedgley et al. (2005b) and interestingly, irrigation was performed with a non-antimicrobial irrigant. The rational of the authors was to assess the “mechanical efficacy of irrigants when delivered at different distances from the apex in the removal of bacteria”. However, other studies have shown that an increase in volume does little to improve the flushing action (van der Sluis et al., 2006). In the present study, the tip of the irrigating needle was placed within 2 mm from the apex and 5 ml of irrigant was introduced into the root canal. Even with the use of an antimicrobial irrigant in the form of 4% sodium hypochlorite, the canals could not be rendered bacteria-free, and this is most likely because the bacteria were exposed to sodium hypochlorite for only 1 min and only a small volume of 5 ml was used.
4.2 Comparing sonic activation of 0.9% saline and 4% sodium hypochlorite

When using the EndoActivator® to induce sonic activation of the irrigants, the group irrigated with sodium hypochlorite experienced a higher percentage kill of 90% ± 16% compared with 80% ± 23% for saline. In this study, the EndoActivator® was set at maximum power, which was 10,000 cycles per minute (166 Hz). It can be deduced from the SEM images of sonic activation of saline that the hydrodynamic shear forces resulting from acoustic streaming are relatively small (Figures 19 and 20). The biofilm is not thick and composed of multiple layers as before, but it is still covers a significant portion of the radicular dentine. The combination of sonic activation with sodium hypochlorite resulted in much more disruption of the dense biofilm, with only a few bacterial cells evident on the radicular dentine surface (Figures 21 to 24).

While none of the SEM images showed the presence of \textit{E. faecalis} within the dentinal tubules, the number of CFU's reflects the presence of bacteria despite the relatively clean radicular dentinal walls. Due to the imprecise nature of the way the teeth were notched and split into halves for visualisation of the root canal, it was impossible to obtain images of the longitudinal sections of all the different treatment groups. However, some of the sections yielded areas which could be imaged under SEM, albeit they were mainly of \textit{E. faecalis} that had invaded the tubules superficially. Since the diamond disc had been used to notch the teeth from the cementum side, most of the canals proximal to the cementum side were covered with dentinal debris. As such, this rendered it impossible to determine if any \textit{E. faecalis} cells penetrated this far into the dentinal tubules. Nevertheless, based on the percentage kill rate (Table 1), it can be deduced that acoustic streaming, even with an antimicrobial irrigant, is insufficient in eradicating bacteria that have invaded the dentinal tubules. It can be postulated that the acoustic energy generated by the sonic instrument results in limited penetration of the irrigant into the dentinal tubules. However, research into this is still in its infancy, and more investigation into this area is warranted.

4.3 Comparing laser activation of 0.9% saline and 4% sodium hypochlorite

This difference between the two irrigants was more marked when the CFU counts for teeth that received laser activation of saline and sodium hypochlorite were observed; the
group that had been irrigated with sodium hypochlorite had achieved a mean percentage kill of 99.93% ± 0.14% compared with the saline group, 73% ± 21%. Although much of the biofilm had been disrupted following activation of the saline irrigant (Figures 25 and 26), a significant number of *E. faecalis* cells were still residing within the dentinal tubules (Figures 27 to 30). It is thus evident that despite the ability of laser mediated cavitation to eradicate bacteria on the surface, unless coupled with an antimicrobial agent, it was not possible to eliminate the bacteria that had invaded the dentinal tubules. Laser activation of sodium hypochlorite, on the other hand, was able to eliminate almost all of the bacteria, and the SEM images showed not only clean radicular dentinal walls (Figures 31 and 32), but also dentinal tubules that were devoid of bacteria (Figures 33 to 35). It is evident that the higher level of energy imparted by the Er,Cr:YSGG laser was able to permit greater penetration of the sodium hypochlorite into the dentinal tubules to cause more effective eradication of the *E. faecalis* bacterial cells. The consistency of the results, whereby laser activated irrigation was able to achieve percentage kills of almost 100% provides more evidence of the efficacy of this method of irrigation.

The use of extracted teeth and the root canals instead of cellulose nitrate membrane filters or dentine blocks in this study was to replicate the *in-vivo* environment, albeit a simplified model. Nonetheless, the teeth used in this study had relatively simple anatomy. Future directions may include the use of root canals with more complex anatomy, such as the mesial roots of lower molars. The presence of isthmuses and fins will test the ability of laser activated irrigation to clean these normally inaccessible areas, and may provide greater insight into the effectiveness of such an irrigation protocol.

Studies that investigated the mechanism of laser energy mediated cavitation effects have shown that the phenomenon is brought about by the rapid implosion of a vapour bubble (Blanken *et al.*, 2009; de Groot *et al.*, 2009). Visualisation of the generation and subsequent implosion of vapour cavities have provided a comprehensive understanding of the ways in which laser activation of anti-bacterial irrigants can aid in debridement of the root canal system. However, most of these studies have visualised these mechanisms at work in plastic containers or glass capillary tubes that simulate a root canal (Blanken *et al.*, 2009; de Groot *et al.*, 2009). Containers or tubes do not share similar dimensions to the small and confined space of the root canal system. It is thus assumed that the pattern of vapour cavity generation and implosion would be duplicated when the irrigant is activated in a similar manner within the root canal. However, we cannot be sure if the presence of a non-uniformly shaped canal, with various fins and isthmuses and the apical foramen, may cause a shift in fluid dynamics, thereby dampening the effectiveness of these mechanisms.
A total of 58 teeth were used in this study. However, the percentage kill results for 10 root canal samples from one of the flow cells could not be calculated (Appendix 1 – Flow cell 5). This was because no detectable bacteria could be cultivated from the root canal of the tooth treated with syringe irrigation of saline. This may have been due to an operator error during serial dilution or culturing of the bacteria. Nonetheless, the results of the viable count showed consistency of the number of colony forming units/ml with the other flow cells. There was a detectable trend, whereby the use of sodium hypochlorite resulted in lower bacterial numbers, and that the use of laser activated irrigation provided the greatest reduction in bacterial cells.

4.4 Clinical considerations

The concentration of sodium hypochlorite used in this study was relatively high (4%), and may have contributed quite significantly to the elimination of *E. faecalis*. The cytotoxic nature of sodium hypochlorite is a concern, and can elicit a severe reaction when extruded out through the apex of the root canal (Hulsmann and Hahn, 2000). Undoubtedly, the potential for extrusion of sodium hypochlorite is increased with the use of the Er,Cr:YSGG laser (George and Walsh, 2008). It is important also to note that their results revealed that most significant factor that determined the amount of extrusion was the size of the apical foramen. It thus reinforces the importance of maintaining instrumentation within the root canal system, the need to respect and preserve the apical constriction as much as possible (Ricucci, 1998), and that the laser be used judiciously to prevent the occurrence of a hypochlorite incident.

In this study, the apices of the root canals were blocked with Cavit and nail varnish prior to irrigation and activation of the irrigant. This creates an apical air lock, which has been shown to reduce the efficacy of hand irrigation. The occlusion of the apices also limited the forward expansion of the vapour bubble generated by the laser, and prevented the expulsion of the irrigant out of the root canal, especially as these canals had been prepared with a size #40 (0.06 taper) instrument to 1 mm beyond the apex. Blanken *et al.* (2009) have shown that within an artificial root canal within a glass block, the limited lateral and forward expansion within the confines of the root canal resulted in the generation of primary and secondary cavitation bubbles. The cavitation effect subsequently translates into the generation of shear forces against the canal walls which produced the results shown in the present study, i.e. disruption of the biofilm and eradication of the *E. faecalis* cells when used in conjunction with sodium hypochlorite.
It should be noted that the power setting in the George and Walsh (2008) study was 1.25W at 20 Hz; the Er,Cr:YSGG laser in our study was set at one-fifth of that employed in their investigation. The Er,Cr:YSGG laser was set at 0.25W at 20 Hz. Also, to mimic a clinical situation where minimisation of extrusion would be ideal, only 4 mm of the tip was inserted into the canal and withdrawn slowly while the laser was being activated. The power setting of 0.25W used in the present study was the lowest possible setting for the Waterlase unit. New software upgrades and the new Waterlase MD models now allow for a lower power setting of 0.10W. Thus, a protocol which ensures efficacious debridement of the root canal will require investigation into different permutations of irrigant concentration and power setting to achieve the maximum disinfection with minimum risk of morbidity to the patient. Other factors such as the length of time in which the irrigant should be activated, the type(s) and/or combination of irrigant(s), and the volume of irrigant used should also be established. Ideally, a large volume of irrigant should be activated for as long as possible. However, there are practical clinical considerations such as treatment time, as well as safety issues. Further research into the potential use of lower concentrations of sodium hypochlorite, in combination with different power settings of the Er,Cr:YSGG laser are currently underway.

4.5 Limitations of the study

The ability of *E. faecalis* to persist in an endodontically treated root canal is testimony to its survivability. It has been shown to be capable of withstanding protracted periods of time within the root canal, maintaining their viability for up to 12 months without nutrition (Sedgley *et al*., 2005a). Figdor *et al*. (2003) have shown it to be able to survive in water for more than 4 months, with the viable cell population being maintained at about $10^3$ CFU/ml. These studies reflect the conditions within an instrumented and obturated root canal. In the present study however, the biofilm was constantly bathed in Todd Hewitt Broth and thus received a constant supply of nutrients. Many studies have shown that the capacity for antimicrobial agents in endodontic treatment to exert its effects is dependent upon the physiological state of the bacteria. Portenier *et al*. (2005) examined the susceptibility of *E. faecalis* to 0.0001% sodium hypochlorite when it was in exponential growth phase, stationary phase and starvation phase. The results showed that the resistance of *E. faecalis* was highest when the cells were in the starvation phase, with more than 4% of the bacterial cells surviving after 10 min of exposure to sodium hypochlorite. When compared against *E. faecalis* cells that were in the exponential growth
phase or the stationary phase, the starved cells were able to survive in numbers 1,000 to 10,000 times higher following exposure to sodium hypochlorite. Cells that were growing were the most sensitive to the antimicrobial agent (Portenier et al., 2005).

Liu et al. (2010) also investigated the ability of *E. faecalis* cells to form biofilms when in starvation phase, and compared against cells in the exponential growth phase and stationary phase. The results showed that starved *E. faecalis* cells were capable of biofilm formation, albeit slower as compared to cells in the other two phases. When the biofilm was subject to sodium hypochlorite exposure, cells in the starvation phase were more resistant to killing by 5.25% sodium hypochlorite. The resistance of *E. faecalis* biofilm to sodium hypochlorite in the starved state also increased with maturation of the biofilm, as the 48 hour-old biofilm showed significant lower reduction of viable cells compared to those of the 6 hour and 24 hour-old biofilm (Liu et al., 2010). Interestingly, their results also showed that *E. faecalis* cells in the exponential growth phase had lower susceptibility to sodium hypochlorite when compared against cells in the stationary phase. This is in contrast to the findings of Portenier et al. (2005). Despite the conflicting evidence, the elevated resistance of *E. faecalis* cells in the starvation phase must account for its ability to persist within the root canal following therapy. Thus, the use of cells in the starvation phase within our current experimental protocol is an avenue for further research.

The method with which the teeth were sampled was highly effective in recovering most, if not all, of the bacteria within the root canal, including those that were residing within the dentinal tubules. This method would also allow for sampling of root canals with anatomy more complex than those used in this study, where fins, anastomoses and isthmuses exist. The shortcoming of paper point sampling is the inability to access these sites (Sundqvist et al., 1998). Molander et al. (1998) also noted that in retreatment cases, particles of remnant gutta percha may preclude sampling fluid from areas where bacteria were present, thereby leading to a false negative result. However, this technique of sampling is destructive, and cannot be applied in *in vivo* studies. Care was also taken to ensure that the bacteria sampled would be from within the root canal, as the bacteria biofilm that may have developed on the surface of the root would have been eliminated by soaking the entire tooth in 4% sodium hypochlorite for 1 hour. A previous study had shown that this regime would result in 99.99% kill of *E. faecalis* (Plutzer, 2009).
Chapter 5. Conclusion

Sonic and laser activated irrigation, in conjunction with the use of an antimicrobial irrigant, resulted in reduction of *E. faecalis* cells from within the root canal. The results were particularly marked when the Er,Cr:YSGG laser was used to activate a 4% sodium hypochlorite solution. This superior ability to disinfect the root canal system predictably may potentially remove the need for the placement of an inter-appointment intra-canal medicament. It would be of interest to determine if the application of sonic and laser activated irrigation would be capable of reducing bacterial numbers to levels where calcium hydroxide had been placed as an intra-canal medicament. Well-controlled prospective clinical studies are necessary to determine if the microbial load following ultrasonic activation within a single visit endodontic treatment would be comparable to that after medicament placement. Long term evaluation of the outcome would also be essential to determine the implications of sonic and laser activated irrigation in the healing of post-treatment apical periodontitis.

There is mounting evidence to support the application of single visit endodontics. Apart from the similar success rate to two visit endodontic therapy, and the lack of “flare ups” and post operative pain (Oliet, 1983; Trope, 1991), there are the practical concerns that need to be addressed. To date, there are no studies which have compared the outcomes of single visit against multiple visit root canal retreatment. In a vast country like Australia, many patients who require endodontic therapy often live outside of the metropolitan areas and the commute to receive treatment can involve hours of travel. This may not be feasible for many patients. There are also patients who, due to physical impediments, may find it difficult to access treatment should it require several appointments. This should, however, not be a reason to deprive such patients of the right to receive endodontic therapy, and the right to preserve their dentition. These reasons reinforce the convenience of single visit endodontic therapy, and from a practicality standpoint, a more viable option for some of our patients. The persistence of bacteria such as *E. faecalis*, which have evolved to maintain their viability in spite of the antimicrobial measures employed in endodontic therapy, presents a significant problem in ensuring the eradication of these bacteria and thus eliminating the post-treatment disease.

It is without doubt that the present study will require much refinement and further research into laser activated irrigation to maximise the potential of this technology. However, it is hoped that this will pave the way for the possible institution of a single visit
treatment protocol which can predictably eliminate \textit{E. faecalis} and other bacteria so as to ensure the most favourable treatment outcome.
Chapter 6. References


Ike Y, Clewell DB. 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. J Bacteriol 1992: 174: 8172–8177.


Trope M, Bergenholtz G. Microbiological basis for endodontic treatment: can a maximal outcome be achieved in one visit? Endod Topics 2002; 1:40-53.


Appendix 1

Each flow cell was incubated with *E. faecalis* for a period of 4 weeks before the root canal samples were subjected to the different treatment regimen. Serial dilution was performed to attain the number of colony forming units/ml. The percentage kill was calculated by dividing the treatment group against the positive control which was syringe irrigation with saline. The values in italics were disregarded in the results.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>CFU/ml</th>
<th>Mean</th>
<th>% Left</th>
<th>% kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.11E+07</td>
<td>1.11E+07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>6.15E+06</td>
<td>4.00E+06</td>
<td>5.08E+06</td>
<td>46%</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>8.38E+06</td>
<td>2.86E+06</td>
<td>5.62E+06</td>
<td>51%</td>
</tr>
<tr>
<td>NaOCl</td>
<td>1.00E+07</td>
<td>1.00E+07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>6.25E+05</td>
<td>4.05E+05</td>
<td>5.15E+05</td>
<td>5%</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>2.50E+02</td>
<td>0.002%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>CFU/ml</th>
<th>Mean</th>
<th>% Left</th>
<th>% kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.38E+07</td>
<td>1.38E+07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>7.10E+05</td>
<td>2.95E+05</td>
<td>5.03E+05</td>
<td>4%</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>3.80E+06</td>
<td>9.20E+06</td>
<td>3.25E+06</td>
<td>39%</td>
</tr>
<tr>
<td>NaOCl</td>
<td>6.15E+05</td>
<td>2.80E+06</td>
<td>1.71E+06</td>
<td>12%</td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>3.60E+04</td>
<td>8.00E+03</td>
<td>2.20E+04</td>
<td>0.159%</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>0.00E+00</td>
<td>3.50E+02</td>
<td>1.75E+02</td>
<td>0.001%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 3</th>
<th>CFU/ml</th>
<th>Mean</th>
<th>% Left</th>
<th>% kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.44E+04</td>
<td>3.44E+04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>7.95E+04</td>
<td>8.60E+04</td>
<td>7.05E+04</td>
<td>229%</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>4.85E+03</td>
<td>4.55E+03</td>
<td>4.25E+03</td>
<td>13%</td>
</tr>
<tr>
<td>NaOCl</td>
<td>1.72E+04</td>
<td>1.72E+04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>6.70E+03</td>
<td>1.17E+04</td>
<td>1.63E+04</td>
<td>34%</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>3.50E+01</td>
<td>2.25E+02</td>
<td>3.50E+01</td>
<td>9.83E+01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 4</th>
<th>CFU/ml</th>
<th>Mean</th>
<th>% Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>9.45E+04</td>
<td>9.00E+04</td>
<td>9.23E+04</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>9.05E+03</td>
<td>1.31E+04</td>
<td>1.11E+04</td>
</tr>
<tr>
<td>NaOCl</td>
<td>7.40E+03</td>
<td>7.40E+03</td>
<td></td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>1.55E+02</td>
<td>2.95E+02</td>
<td>2.25E+02</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>3.50E+01</td>
<td>0.00E+00</td>
<td>1.75E+01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 5</th>
<th>CFU/ml</th>
<th>Mean</th>
<th>% Left</th>
<th>% kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.09E+05</td>
<td>1.52E+05</td>
<td>2.31E+05</td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>1.92E+04</td>
<td>2.46E+04</td>
<td>2.19E+04</td>
<td>10%</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>2.04E+04</td>
<td>9.50E+03</td>
<td>1.50E+04</td>
<td>6%</td>
</tr>
<tr>
<td>NaOCl</td>
<td>3.80E+04</td>
<td>5.60E+04</td>
<td>4.70E+04</td>
<td>20%</td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>9.00E+02</td>
<td>4.50E+02</td>
<td>6.75E+02</td>
<td>0.293%</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>4.00E+01</td>
<td>1.00E+01</td>
<td>2.50E+01</td>
<td>0.011%</td>
</tr>
</tbody>
</table>
## Appendix 2

Results of the protein assay and calculations performed to obtain the CFU/μg. Many of the samples returned values that were too low to be detected, and as such, the results were expressed as CFU/ml, rather than CFU/μg.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>CFU/ml</th>
<th>Mean CFU/ml</th>
<th>Protein ug/ml</th>
<th>CFU/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>1.11E+07</td>
<td>1.11E+07</td>
<td>2.18</td>
<td>5.07E+06</td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>6.15E+06</td>
<td>4.00E+06</td>
<td>5.08E+06</td>
<td>2.33</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>8.38E+06</td>
<td>2.86E+06</td>
<td>5.62E+06</td>
<td>undetectable</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>1.00E+07</td>
<td>1.00E+07</td>
<td>undetectable</td>
<td></td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>6.25E+05</td>
<td>4.05E+05</td>
<td>5.15E+06</td>
<td>undetectable</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>2.50E+02</td>
<td>4.41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>CFU/ml</th>
<th>Mean CFU/ml</th>
<th>Protein ug/ml</th>
<th>CFU/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>1.38E+07</td>
<td>1.38E+07</td>
<td>2.48</td>
<td>5.56E+06</td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>7.10E+05</td>
<td>2.95E+05</td>
<td>5.03E+05</td>
<td>3.99</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>3.80E+06</td>
<td>9.20E+06</td>
<td>3.25E+06</td>
<td>undetectable</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>6.15E+05</td>
<td>2.80E+05</td>
<td>1.71E+06</td>
<td>undetectable</td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>3.60E+04</td>
<td>8.00E+03</td>
<td>2.20E+04</td>
<td>3.87</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>0.00E+00</td>
<td>3.50E+02</td>
<td>1.75E+02</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 3</th>
<th>CFU/ml</th>
<th>Mean CFU/ml</th>
<th>Protein ug/ml</th>
<th>CFU/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>3.44E+04</td>
<td>3.44E+04</td>
<td>2.01</td>
<td>1.71E+04</td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>7.95E+04</td>
<td>8.60E+04</td>
<td>7.05E+04</td>
<td>2.55</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>4.85E+03</td>
<td>4.55E+03</td>
<td>4.25E+03</td>
<td>2.46</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>1.72E+04</td>
<td>1.72E+04</td>
<td>1.72E+04</td>
<td>undetectable</td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>6.70E+03</td>
<td>1.17E+04</td>
<td>1.63E+04</td>
<td>1.16E+04</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>3.50E+01</td>
<td>2.25E+02</td>
<td>3.50E+01</td>
<td>9.83E+01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 4</th>
<th>CFU/ml</th>
<th>Mean CFU/ml</th>
<th>Protein ug/ml</th>
<th>CFU/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>not detected</td>
<td></td>
<td>2.79</td>
<td></td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>9.45E+04</td>
<td>9.00E+04</td>
<td>9.23E+04</td>
<td>2.46</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>9.05E+03</td>
<td>1.31E+04</td>
<td>1.11E+04</td>
<td>3.817</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>7.40E+03</td>
<td>7.40E+03</td>
<td>undetectable</td>
<td></td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>1.55E+02</td>
<td>2.95E+02</td>
<td>2.25E+02</td>
<td>undetectable</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>3.50E+01</td>
<td>0.00E+00</td>
<td>1.75E+01</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 5</th>
<th>CFU/ml</th>
<th>Mean CFU/ml</th>
<th>Protein ug/ml</th>
<th>CFU/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td>3.09E+05</td>
<td>1.52E+05</td>
<td>1.87</td>
<td>1.23E+05</td>
</tr>
<tr>
<td>Saline + sonic</td>
<td>1.92E+04</td>
<td>2.46E+04</td>
<td>2.19E+04</td>
<td>1.9</td>
</tr>
<tr>
<td>Saline + laser</td>
<td>2.04E+04</td>
<td>9.50E+03</td>
<td>1.50E+04</td>
<td>1.59</td>
</tr>
<tr>
<td><strong>NaOCl</strong></td>
<td>3.80E+04</td>
<td>5.60E+04</td>
<td>4.70E+04</td>
<td>2.41</td>
</tr>
<tr>
<td>NaOCl + sonic</td>
<td>9.00E+02</td>
<td>4.50E+02</td>
<td>6.75E+02</td>
<td>undetectable</td>
</tr>
<tr>
<td>NaOCl + laser</td>
<td>4.00E+01</td>
<td>1.00E+01</td>
<td>2.50E+01</td>
<td>undetectable</td>
</tr>
</tbody>
</table>