Efficacy of laser and ultrasonic activated irrigation on eradicating a mixed species biofilm grown in the mesial roots of human mandibular molars

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

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Table of Contents

Table of Figures

Table of Tables

Abstract

Aim

To compare the efficacy of Er,Cr:YSGG laser and ultrasonic activated irrigation on eradicating a biofilm grown in the mesial roots of human mandibular molars.

Methods

A biofilm containing *Enterococcus faecalis*, *Streptococcus sanguinis* and *Fusobacterium nucleatum* was grown over 4 weeks in the mesial root canals of decoronated human mandibular molar teeth. Following removal from the flow cell, control roots (n=5) received no further treatment. The remaining tooth roots were chemomechanically prepared using different irrigating protocols: saline standard irrigation (Saline SI; n=15); 4% NaOCl and 15% EDTAC with ultrasonic activated irrigation (UAI; n=18); 4% NaOCl and 15% EDTAC with laser activated irrigation using power settings 0.5 W (LAI 0.5 W; n=18) or 0.75 W (LAI 0.75 W; n=10). Following treatment and crushing, bacteria were quantified by culturing (CFU/mL) and quantitative real-time PCR (qPCR). One tooth from each group was subjected to SEM analysis.

Results

Quantification by culturing revealed significant differences between controls and all other treatment groups. Significant differences were found between Saline SI and UAI, Saline SI and LAI 0.5 W and also between LAI 0.5 W and LAI 0.75 W. No significant differences were found between Saline SI and LAI 0.75 W or between UAI and LAI 0.5 W or LAI 0.75 W.

From qPCR results, significant differences were found between controls and all other treatment groups. No statistically significant differences were found between Saline SI and UAI, LAI 0.5 W or LAI 0.75 W.

Conclusions

Both culture and molecular techniques showed that mechanical preparation significantly reduces bacteria from the root canals of lower molar mesial roots. Further reductions were achieved by irrigating with 4% NaOCl and 15% EDTAC UAI or 4% NaOCl and 15% EDTAC LAI. No significant reductions in bacterial number were found between UAI and LAI protocols.

Declaration

I, Jonathan Race, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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1 Introduction

Apical periodontitis is inflammation of the periapical tissues and is most often caused by microbial infection of the root canal system (Kakehashi *et al.*, 1965; Sundqvist, 1976; Möller *et al.*, 1981). Once infection of the root canal system occurs, the host responds in a coordinated manner to prevent further microbial invasion into the surrounding tissues (Cotti *et al.*, 2014). The complex anatomy of the root canal system often prevents the host from successfully eliminating the microorganisms (Vera *et al.*, 2012), with the disease usually persisting once the infection has become established as a biofilm (Siqueira *et al.*, 2012). Endodontic treatment at this point is aimed at removing the causative agents and preventing reinfection, thereby providing suitable conditions for the periapical tissues to return to health.

To remove microorganism from the root canal system, treatment has been mainly centered around mechanical instrumentation, irrigation with an antimicrobial and tissue dissolving agent and the judicious use of intraradicular medicaments between appointments. Currently it is not possible to predictably eliminate all bacteria from the root canal system (Paiva *et al.*, 2013a; Rôças *et al.*, 2013) and so healing remains unpredictable (Ng *et al.*, 2011). New treatment approaches are necessary to improve the removal of bacteria from the root canal system, especially in teeth with complex anatomical features such as those found in the mesial roots of lower mandibular molars (Nair *et al.*, 2005). To improve the efficacy of traditional chemomechanical procedures, it has been suggested that ultrasonic and laser activated irrigation protocols may be used (Harrison *et al.*, 2010; Peters *et al.*, 2011).

1.1 Microbial basis of endodontic disease

Bacterial invasion and colonisation of healthy sterile dental pulp tissues was shown by (Kakehashi *et al.*, 1965) to cause pulp necrosis with continued infection of the root canal system. This was demonstrated by experimentally exposing the pulp tissues of both germ-free (gnotobiotic) and conventional rats (with a normal complex microbiota). The pulpal tissues of all conventional rats became necrotic and the periapical tissues were chronically inflamed with abscess formation. In stark contrast, none of the pulps of germ-free rats were devitalised or abscessed and minimal inflammation was seen. Signs of healing were seen

with the formation of a reparative dentine bridge over vital pulp tissue. The causal relationship between bacteria and apical periodontitis was not fully established by Kakehashi *et al.* (1965) as it was possible that although bacteria were present, the cause of the apical periodontitis may have been due to endogenous necrotising pulp tissue rather than the intraradicular bacteria.

Sundqvist (1976) radiographically examined traumatised non carious teeth with intact pulp chambers before the pulp chambers were accessed for microbiological sampling and anaerobic and aerobic culturing. Nineteen teeth had periapical radiolucencies and all but one (possibly a false negative result) was culture positive whereas samples taken from the remaining 13 teeth without periapical radiolucencies were culture negative. Such clear differences between the groups showed that bacteria may invade the root canal system of traumatised teeth with intact pulp chambers and establish an intraradicular infection. Once bacterial colonisation of the root canal has occurred, there is a high chance (although not inevitable) that radiographically determined apical periodontitis will develop whereas if necrotic pulpal tissue remains sterile, no periapical pathosis will occur.

The role of bacteria in causing apical periodontitis was further established by Möller *et al.* (1981) who aseptically necrotised the pulp tissue of 78 teeth in nine monkeys. Fifty-two pulp chambers were infected by indigenous bacteria from the Monkey's saliva, while 26 were kept sterile. After six months, clinical, radiographic and histopathological examination revealed that all teeth with necrotic pulps that were sealed immediately after pulp amputation remained sterile and did not induce inflammatory reactions in the apical tissues. In contrast, 12 teeth that were infected with indigenous bacteria showed clinical signs of infection, 47 teeth showed radiographic signs of periapical resorption and all 52 infected teeth showed histological signs of a strong inflammatory reaction in the periapical tissues. Möller *et al.* (1981) clearly demonstrated that uninfected necrotic pulpal tissue does not cause periapical pathosis and that the risk of haematogenous infection of the root canal system over a six month period is very low. When bacteria enter and colonise the root canal system with a necrotic pulp, a strong inflammatory reaction occurs in the periapical tissues. This reaction may often be clinically and radiographically observed.

1.1.1 Identification of endodontic microbiota

1.1.1.1 Microbial sampling of endodontic microbiota

The microbial composition, diversity, species richness and abundance has been investigated using culturing and molecular methods. Before either culturing or molecular methods can be used to identify endodontic microbiota, the endodontic infection must first be sampled. Paper point sampling (Byström & Sundqvist, 1981) and modifications of this process (Paiva *et al.*, 2013a), dentine shavings (Krithikadatta *et al.*, 2007; Neelakantan *et al.*, 2014) or crushed tooth samples (Seet *et al.*, 2012) are the main microbial sampling methods used.

The principles of endodontic treatment such as the need for rubber dam isolation, mechanical preparation, irrigation, medication and obturation of the root canal system have been derived from clinical studies using microbial paper point sampling (Byström & Sundqvist, 1981; 1983; 1985; Sjögren *et al.*, 1991; Dalton *et al.*, 1998; Shuping *et al.*, 2000; Card *et al.*, 2002). Paper point sampling is relatively quick and easy to perform while being cost effective; however there are a number of obvious limitations to this method.

Paper points passively sample only the main root canal contents. To increase the recovery of bacteria from dentine tubules or thick biofilm layers, endodontic files have been used to scratch the dentine wall before inserting the paper point into the canal (Paiva *et al.*, 2013a). These methods only sample a very small proportion of the dentine wall and bacteria in dentine tubules that are close to the canal wall. To overcome this problem, canal wall shavings have been collected using different sized burs (e.g. Gates Glidden) however only the main canal wall in straight sections of the canal can be sampled.

Considerable amounts of bacteria may be present in areas such as lateral and accessory canals, fins, anastomoses, ramifications, dentine tubules or adherent biofilm (Sathorn *et al.*, 2007). These anatomical features are particularly prevalent in multi-rooted teeth such as mandibular molar mesial roots (Vertucci, 1984). Nair *et al.* (2005) and Vera *et al.* (2012) both showed bacterial biofilms remained within inaccessible recesses of mandibular molar mesial roots after traditional chemomechanical preparation of the main root canal.

The inability to sample all bacteria from the infected root canal system increases the

likelihood of false negative results occurring. Crushing or pulverising the teeth may improve the detection and quantification of bacteria from infected root canal systems however this method is only practical for *ex-vivo* based studies (Akpata & Blechman, 1982; Miller & Baumgartner, 2010; Seet *et al.*, 2012). The limitations of microbial sampling need to be careful considered before conclusions are made and the findings extrapolated to the clinical setting.

The limitations of microbial sampling methods are similar for both culture and molecular based techniques, however the main advantage or perhaps limitation of some PCR based methods is that bacteria can be dead at the time of processing unless areas such as gene expression or viability are being investigated.

1.1.1.2 Culturing methods

Culturing techniques have been used for many years to investigate the microbial composition and quantity in infected root canal systems. Culturing techniques have also been successfully used to study many different aspects of microbial physiology, pathogenicity and antimicrobial susceptibility. With this understanding has come the development and implementation of many different types of treatment modalities to manage endodontic infections (Siqueira & Rôças, 2005b).

A deeper understanding of the endodontic microbiota came about within a relatively short period of time because of methodological and technological advances. Möller (1966) revolutionised endodontic sampling and culturing techniques by showing the importance of avoiding contamination and the significance of using strict methodology, inactivating agents, paper point sampling and transport media. The importance of anaerobic culturing techniques was also shown to greatly increase the number of bacteria species isolated from the infected root canal (Wittgow & Sabiston, 1975). The ease, sensitivity and standardisation of anaerobic culturing techniques were greatly improved by methodological and technological refinements such as the use of the anaerobic glove box (Aranki & Freter, 1972). These improvements along with the use of the "Anaerobic Laboratory Manual" (Holdeman & Moore, 1972), the judicious visual inspection of colonies (Kantz & Henry, 1974) and the use of a large array of biochemical tests (Jacinto *et al.*, 2003), all led to the belief that a select "species set" was involved in the

pathogenesis of apical periodontitis (Sundqvist, 1992).

1.1.1.3 Limitations of culturing techniques

There are many inherent limitations involved in recovering and identifying bacteria from infected root canals using culturing based approaches. These limitations are as follows: culturing may be expensive, time-consuming and laborious; bacteria may be slow growing; growth may be inhibited by an incomplete understanding of the factors required by particular bacteria or the microbial community; fastidious anaerobic bacteria may be very difficult to grow and identify; culturing techniques are heavily dependent on the experience and knowledge of the microbiologist in performing an assay as sampling and transportation errors may occur. It is often impossible to cultivate and identify large numbers of bacterial species at once (Siqueira & Rôças, 2005b).

Many microbial species do not survive the sampling process. In particular, transportation of samples from *in vivo* to the laboratory for culturing is problematic. By using a reduced transport medium, the survival of anaerobic bacteria sampled *in vivo* is significantly improved (Möller, 1966). Möller (1966) also showed that at least two weeks of incubation is needed to give a true representation of slow growing fastidious microorganisms. From a microbiological perspective, this is both costly and time-consuming (Sathorn *et al.*, 2007).

1.1.1.4 Quantification of endodontic bacteria

When culturing methods are used, bacteria are predominantly quantified by plating a sample with a known volume (e.g. 100 µL) onto a nutrient agar plate and counting the number of colony-forming units (CFU) after incubation. Because the appearance of a colony is recorded, it is not known whether the colony arose from one cell or 1000 cells. Hence, CFU is often expressed as either CFU/mL for liquids and CFU/g for solids.

Culture based studies have a target species detection limit of about 10^3 and 10^4-10^5 using selective and nonselective media respectively (Siqueira & Rôças, 2003; Boutaga *et al.*, 2005; Siqueira & Rôças, 2005b) and varies between species (Boutaga *et al.*, 2005). For bacteria to be quantified they must survive serial dilution, incubation and plating. They

must also multiply at a rate high enough to be observable within the culturing time frame. Some bacteria species may survive these procedures but are unable to be quantified because they are capable of entering a viable non culturable state (Trevors, 2011).

1.1.2 Polymerase chain reaction (PCR)

Molecular technologies began being utilised in the late 1990's. Since that time, there have been many advances in microbial molecular diagnostics, the most prolific being polymerase chain reaction (PCR) technology and its derivatives such as real-time PCR.

1.1.2.1 Basic PCR protocols

The PCR process requires three fundamental sequential steps be repeated multiple times in a thermocycler (Kolmodin & Williams, 1997). To begin the PCR assay, DNA double helix strands are denatured into single stranded nucleotide polymers by heating the reagent to approximately 95°C. Rapid cooling of the reagent to approximately 55°C allows short single-stranded DNA primers to bind (anneal) to their complementary regions on the previously separated DNA template strands. Finally, the temperature of the PCR reagent is quickly raised to approximately 72°C and held for a sufficient period of time to allow a heat resistant thermostable DNA polymerase to bind to the DNA primer. DNA polymerase then begins matching each template DNA base with a corresponding deoxynucleotide (dNTP's) until a double strand of target DNA is made (Miller & Cunningham, 1999).

The power of PCR to amplify a few target molecules (such as DNA from a single cell) to detectable levels is based on the number of cycles the PCR reagent is subjected to (Halliday, 2010). Most PCR assays require 20-40 cycles be performed. Theoretically, each time the cycle is repeated, there is a doubling of the amount of target DNA (Nadder & Langley, 2001). In reality the amplifying power is not this efficient and is self-limiting with amplification factors generally being between 10^5 and 10^9 fold (Kolmodin & Williams, 1997).

Many advances have been made over the last 20 years which has resulted in the widespread use of this technology. Many commercial kits have been developed which have improved the speed, accuracy and cost effectiveness of using this method. Although the

PCR concept is relatively simple, this molecular technique is highly sensitive and requires many components to be optimised and standardised to ensure meaningful and comparable results are achieved (Sedgley *et al.*, 2005a; Brundin *et al.*, 2010; Debnath *et al.*, 2010).

Considerable variation may exist between PCR runs because of minor differences in methodology such as slight pipetting inaccuracies that may or may not be avoidable (Stirling, 2003; Doak & Zaïr, 2012). Many other more important factors such as primer design, the thermostable DNA polymerase used or inhibitory substances may lead to vastly different results (Pavlov *et al.*, 2004; Vianna *et al.*, 2006; Weile & Knabbe, 2009). The experience and wisdom of the researchers to perform and optimise the PCR is an important variable which is hard to quantify and rarely qualified in any study.

1.1.2.2 Methodological detection limits

Most molecular studies of endodontic infections using rudimentary PCR or quantitative PCR have an analytical sensitivity of about 10 to 100 cells (Sedgley *et al.*, 2005c) however the actual sensitivity may be more in the order of 10^2 to 10^3 cells when dilution factors using small aliquots (5-10%) of the whole sample are used (Siqueira & Rôças, 2003; 2005b).

1.1.2.3 Advantages of PCR based studies

The limitations of culturing were not truly realised until molecular techniques began to be used to study endodontic microbiota (Siqueira & Rôças, 2005b). Conrads *et al.* (1997) was the first to use PCR techniques to detect bacteria from infected root canals that were difficult to differentiate after culturing. Applying this new technology to the study of known bacteria with fastidious growth requirements, it was soon realised that many difficult to cultivate bacteria were highly prevalent in primary endodontic infections. For example, Spirochaetes had been observed in endodontic infections using light microscopy however only PCR methods showed that *Treponema denticola* was present in approximately 80% of canals (Rôças *et al.*, 2003).

Compared to culturing based methods, PCR-based technologies have been used to detect an increased prevalence of commonly isolated bacteria in both primary and

persistent/secondary endodontic infections. For example it has been shown that the commonly isolated *Enterococcus faecalis* (*E. faecalis*) species was far more prevalent in persistent/secondary infections than previously revealed by culture based studies (Molander *et al.*, 1998; Zoletti *et al.*, 2006; Gomes *et al.*, 2008). Gomes *et al.* (2005) found commonly isolated bacteria to be between two and six times more prevalent using PCR techniques compared to culturing based methods.

Molecular based methods have also been shown to be advantageous for identifying new phylotypes that have never been cultivated (Siqueira & Rôças, 2009). The importance of these newly identified phylotypes and their role in the pathogenesis of endodontic disease is still yet to be determined. PCR techniques will most likely play an important role in understanding this process. PCR technology has deepened our understanding of endodontic infections by both confirming the findings of culture based studies while also doubling the number of bacteria species found in endodontic infections (Siqueira & Rôças, 2009).

1.1.3 Real-time PCR

Real-time PCR is a variation of the standard PCR technique and is used to quantify DNA or RNA (via a reverse transcriptase) in a sample. The same three basic stages of denaturation, annealing and extension are performed in real-time PCR (Ma, 2005). Real-time PCR differs to traditional PCR in that fluorescent molecules are added to the reaction mixture to bind with the product being amplified by the PCR. The real-time PCR instrument contains both a light source to periodically excite the fluorescent molecules and a detector to capture the fluorophore emissions throughout the thermal cycling process (Logan & Edwards, 2004). As the product is amplified, a corresponding increase in fluorescence is detected. Therefore the amount of fluorescence can be quantified and the rate of amplification determined at any point throughout the PCR, hence the name – "real-time PCR".

A major advantage of using real-time PCR is that bacteria can be quantified at the time of analysis rather than with culturing which requires a number of days or weeks before quantification. Real-time quantitative PCR (qPCR) has been used to quantify both single species and multiple species in a sample (Saito *et al.*, 2009). This technology is extremely useful for quantifying bacteria numbers when culturing results produce negative outcomes

or when fastidious or as-yet uncultivable bacteria are being studied (Rôças & Siqueira, 2012).

The number of bacteria in endodontic infections is considerably higher in both pre and post treatment samples than initially thought with culturing (Vianna *et al.*, 2006). PCR based studies have shown that current treatment protocols are not as effective as once thought.

1.1.3.1 The chemistry of real-time PCR

There are two main methods used to obtain a fluorescent signal. The first method depends on an increase in fluorescence caused by a conformational change in a fluorescent dye when it binds with double stranded DNA. The second method uses fluorescent resonance energy transfer (FRET). These molecules attach to probes, primers or the PCR product. An increase in fluorescence at a particular wavelength is seen when a specific DNA sequence is amplified (Saunders, 2004).

1.1.3.2 The threshold level

In the initial cycles of the real-time PCR, low DNA strand numbers results in low levels of fluorescence being detected, thereby forming the baseline for future amplification cycles to be compared against. As more amplicon accumulates with each cycle, the fluorescence signal increases with each cycle (Too & Anwar, 2006). The threshold level of the real-time PCR reaction is set at a value at least three standard deviations above the baseline noise level (Saunders, 2004) and as low as possible (VanGuilder *et al.*, 2008).

The threshold level needs to be set within the early exponential phase of the PCR reaction, when all reagents are still in excess, the DNA polymerase is highly efficient when the product is in low concentration. This ensures that there is minimal competition for the primers' annealing capabilities (Saunders, 2004) and is especially important when small initial amounts of DNA are being quantified (Jensen, 2012).

During the initial cycles of the PCR there is an exponential accumulation of template. As the reaction continues, amplification is inhibited due to various factors such as substrate

depletion, loss of enzyme activity and accumulation of amplicon which compete for binding sites. This leads to a plateau phase of amplicon production. Regardless of the amount of starting template, end product analysis will show similar amounts of amplified template (Too & Anwar, 2006). Measuring the fluorescence level in the early amplification phase overcomes the problems of end point analysis and allows the product level to be quantified.

It is important to note that the concentration of amplicon at the threshold level is the same for different samples regardless of the starting concentration (Too & Anwar, 2006). This principle allows different samples to be compared because the varying factor will be the number of cycles that are needed to increase the amount of product and corresponding fluorescence to the threshold level.

1.1.3.3 Threshold cycle and quantification

The cycle number at which point the fluorescence signal crosses the threshold level has commonly been referred to as the threshold cycle (Ct), crossing point (Cp) or take-off point (TOP). These terms have all been coined by competing manufactures of real-time PCR instruments and are not scientifically accurate. Bustin *et al.* (2009) has proposed the term quantification cycle as part of the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" guidelines. These guidelines have been promoted to ensure consistency within the scientific literature. The term quantification cycle has not yet been widely adopted.

The Ct value is inversely related to the amount of starting template. The more template there is to begin with, the fewer the number of cycles needed to reach the Ct. If the log of initial template amounts for a set of dilutions is plotted against Ct values, then a straight line (representing a linear relationship) can be drawn between the plots. From this relationship, the amount of target DNA in a sample may be inferentially determined by either absolute or relative quantification methods (VanGuilder *et al.*, 2008).

This method of quantifying DNA is often referred to as real-time quantitative PCR (qPCR) (Alves *et al.*, 2012).

1.1.3.4 Absolute quantification

Absolute quantification is the term used in qPCR to describe measurements that estimate a target's abundance relative to that of a standard curve (Johnson *et al.*, 2013). Comparing sample Ct values against Ct values of a serially diluted standard (calibrator) of known starting concentration will allow the unknown sample concentration to be quantified. To ensure that the standard curve encompasses a large range, serial ten-fold dilutions in the range of about seven orders of magnitude should be carried out. This process of quantification is only as accurate as the measurements of the serially diluted standard and so rigorous accuracy is needed to normalise the sample data and correct for any tube-to-tube variations (Bustin & Nolan, 2004).

Alternatively, mathematical modelling without the use of standard dilutions has been used to estimate the initial amounts of amplicon (Smith *et al.*, 2007). Variation in PCR efficiency, correlation coefficient (R^2) and deterioration of the calibrator template are all important for absolute quantification (Dhanasekaran *et al.*, 2010).

1.1.3.5 Relative quantification

Relative quantification is another method used to quantify real-time PCR samples and is commonly used to measure changes of a target gene when examined in two different conditions (Too & Anwar, 2006). Relative quantification compares the Ct values for a target gene in one sample (i.e. treated) with the Ct values for the same target gene in another sample (i.e. untreated) (VanGuilder *et al.*, 2008). To monitor experimental variability, an endogenous reference (housekeeping) gene or RNA subunit must be used as a control during the same reaction (Saunders, 2004; Bustin *et al.*, 2009; Jensen, 2012). It is assumed that the expression of this reference gene (or genes) is constant in both treated and untreated samples and is therefore used to normalize the target gene's expression levels in both the treated and untreated samples. The reference gene must have comparable amplification efficiency as the target gene for this calculation to be accurate (VanGuilder *et al.*, 2008).

1.1.4 Limitations of PCR based studies

1.1.4.1 Survival of DNA or RNA within the root canal system

Molecular techniques examining microbial richness and abundance do not require bacteria to be alive at the time of sampling, rather DNA and RNA must only be in a suitable condition to be analysed. PCR analysis of genomic DNA provides a record of bacterial composition at a particular point in time and does not indicate bacterial viability seen with culturing (Siqueira & Rôças, 2005b). It is currently unknown how long DNA and RNA may remain within the intraradicular environment before decomposing, especially if a viable biofilm is present.

A few studies have investigated how long DNA may survive within the root canal environment. Sedgley *et al.* (2005a) showed that intact amplifiable DNA may remain within an obturated tooth for over a year. They inoculated "heat killed" *E. faecalis* into sterile *ex vivo* root canals which were then obturated. After storing the roots in 100% humidity for one year, they found that ribosomal DNA (rDNA) was still present and amplifiable using PCR based techniques.

Brundin *et al.* (2010) showed that DNA from heat killed *E. faecalis* could survive in the root canal environment for over two years however if *E. faecalis* DNA was incubated in either water or 10-50% sera, the DNA decomposed below detection limits. They also found that cell bound DNA was more resistant to decay than 'free' DNA and that DNase produced by *Prevotella intermedia* was able to quickly degrade DNA.

One way of reducing the chance of falsely "counting" dead bacteria is to analyse messenger RNA (mRNA) using reverse transcriptase quantitative PCR (RT-qPCR). Messenger RNA is far less stable than rRNA or DNA and may be used to determine cellular viability (Klatte & Bauer, 2009). Quantitative real-time PCR and RT-qPCR are the most popular methods used for quantifying DNA and RNA respectively (Mayer *et al.*, 2011; Jensen, 2012).

Williams *et al.* (2006) used RT-qPCR to analyse *E. faecalis* mRNA recovered from refractory endodontic infections. Although only four samples were found to have *E. faecalis* in a viable non culturable state, they were able to conclusively show that *E. faecalis* was viable at the time of persistent/secondary infection sampling.

1.1.4.2 Variation in prevalence

Bacterial prevalence varies between PCR studies that use the same basic methodology. The reasons for this are not well formulated however it is probably due to the heterogeneous nature of endodontic infections. Prevalence levels may also vary because the microbial composition may change considerably in response to changes in the intra and extraradicular environment (Fabricius *et al.*, 1982).

Inaccurate prevalence data may lead to incorrect assumptions being made about the pathogenesis of apical periodontitis. Before PCR techniques were widely utilised to study endodontic infections, 44-85% of canals undergoing root canal retreatment were found to be positive for bacterial colonisation (Möller, 1966; Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b). Low bacterial prevalence led researchers to overestimate the influence of non-microbial factors (Nair *et al.*, 1999) causing post treatment apical periodontitis (Siqueira & Rôças, 2005b). Since this time, PCR based studies have shown that almost all canals with post treatment apical periodontitis are positive for bacteria (Rôças & Siqueira, 2012) thereby showing that bacteria are the most likely cause of post treatment apical periodontitis.

1.1.5 The microbiology of endodontic infections

1.1.5.1 Primary intraradicular infections

1.1.5.1.1 The number of species per canal

Many different species have been cultivated from primary intraradicular infections however an average of five strains (range 1-11) are usually cultivated from each canal (Wittgow & Sabiston, 1975; Sundqvist, 1992; Le Goff *et al.*, 1997; Lana *et al.*, 2001). Molecular methods greatly expanded the number of species/phylotypes found in primary affected root canals with an average of 20 species/phylotypes being found in each canal (Munson *et al.*, 2002). The majority of molecular methods used to determine the number of species in a canal do not differentiate between viable and non-viable bacteria rather they give a historical overview of the bacteria present in the canal during the course of the

infection.

1.1.5.1.2 The most common species found in primary intraradicular infections

Culturing based studies have found that although a diverse range of bacteria can be isolated from initially infected root canals, there is a "species set" that are consistently more prevalent than other species. This "species set" is thought to play a central role in the pathogenesis of apical periodontitis (Sundqvist, 1994). The most commonly isolated and culturable species of bacteria from primary intraradicular infections are Gram-negative species that belong to the genera *Fusobacterium*, *Porphyromonas*, *Prevotella*, and *Campylobacter*. Gram-positive anaerobes with the highest prevalence in primary root canal infections are from the genera *Peptostreptococcus, Eubacterium, and Pseudoramibacter*, as well as facultative or microaerophilic streptococci (Siqueira & Rôças, 2005a). PCR based studies have not only confirmed these findings but have also revealed an increase in richness and abundance of other suspected endodontic pathogens, thereby expanding the "species set" to include many as-yet-uncultivated bacteria. There are many more pathogenic phylotypes that are likely to be discovered in the near future using molecular methods (Siqueira & Rôças, 2009).

1.1.5.1.3 Expanding the list of endodontic microbiota

The list of endodontic microbiota has been continually expanding at a rapid rate because of the utilisation of molecular based methods. Broad-range investigations of bacterial communities (Munson *et al.*, 2002; Saito *et al.*, 2006; Sakamoto *et al.*, 2006; Sakamoto *et al.*, 2008) and large scale clinical studies investigating the association and prevalence of bacteria with endodontic disease have had the most significant impact on increasing this number. The diversity of endodontic microbiota was reviewed by (Siqueira & Rôças, 2009) and they reported that 391 bacterial, four fungal and one archaeal taxa have been detected in primary intraradicular infections and this list is increasing in number with the utilisation of newer technologies (Ozok *et al.*, 2012).

1.1.5.2 Persistent/secondary endodontic infections

Chemomechanical preparation followed by an intraradicular medication has been shown

to significantly reduce the number and diversity of bacteria within the root canal system (Byström & Sundqvist, 1981; 1983; Sjögren *et al.*, 1997). Many species able to thrive in primary intraradicular infections are unable to survive within the extremely harsh environment created by professional intervention. Culturing and molecular based studies have generally found that the microbiota of root canal treated teeth with apical periodontitis is qualitatively and quantitatively different from primary intraradicular infections (Molander *et al.*, 1998; Siqueira & Rôças, 2009).

1.1.5.2.1 The number of species per canal

Culture based studies have found that canals with persistent/secondary infection, usually harbour only one or two species, although occasionally this number can be higher (Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b). Studies using species-specific PCR have shown an increased prevalence of suspected pathogens in teeth with failed root canal treatment (Siqueira & Rôças, 2009).

Technically inadequate root filled teeth show an increased number of species compared to teeth with satisfactory root fillings. The infections in technically inadequate root filled teeth resemble primary infections rather than resembling persistent/secondary infections (Rôças *et al.*, 2004a; Siqueira & Rôças, 2004).

1.1.5.2.2 The dominance of Gram-positive species

Both culturing and PCR based studies have shown that persistent/secondary infections are dominated by facultative anaerobes and to a lesser extent by obligate anaerobes. It has also been shown that Gram-positive species form an overwhelming majority in persistent/secondary infections compared to Gram-negative bacteria (Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b; Rôças *et al.*, 2004a; Siqueira & Rôças, 2004).

Enterococcus faecalis has been one of the most commonly isolated species with both culturing and molecular methods (Molander *et al.*, 1998; Sundqvist *et al.*, 1998; Zoletti *et al.*, 2006; Pirani *et al.*, 2008; Rôças & Siqueira, 2012). Other species of bacteria commonly isolated with culturing methods are *Peptostreptococcus, Streptococcus, Lactobacillus,*

Actinomyces and the most common fungi is *Candida albicans* (Molander *et al.*, 1998; Sundqvist *et al.*, 1998) (Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b; Gomes *et al.*, 2004; Gomes *et al.*, 2008) however this list has been expanded by molecular methods (Siqueira & Rôças, 2009).

1.1.6 Three bacteria of importance in endodontic infections

1.1.6.1 *Enterococcus faecalis*

E. faecalis is a facultative anaerobic Gram-positive cocci that lives in vast quantities in the human intestinal lumen and may also survive to a lesser extent in the oral cavity (Koch *et al.*, 2004). *E. faecalis* is an opportunistic pathogen (Dahl & Bruun, 2013) which may infect multiple areas of the body including the root canal system (Rôças & Siqueira, 2012).

1.1.6.1.1 The role of *Enterococcus faecalis* in persistent/secondary infections

E. faecalis is associated with both primary and persistent/secondary infections but is far more prevalent in the latter. Culture based studies have reported *E. faecalis* prevalence in persistent/secondary infections to be between about 20-70% (Möller, 1966; Molander *et al.*, 1998; Sundqvist *et al.*, 1998) (Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b) (Peciuliene *et al.*, 2000; Hancock *et al.*, 2001; Peciuliene *et al.*, 2001) whereas PCR detection methods have shown higher percentages (40-80%) (Pavlov *et al.*, 2004; Rôças *et al.*, 2004a; Zoletti *et al.*, 2006; Gomes *et al.*, 2008; Pirani *et al.*, 2008; Ozbek *et al.*, 2009; Rôças & Siqueira, 2012).

Some culture based studies have found *E. faecalis* to be the only species isolated from canals with failed root canal treatment (Molander *et al.*, 1998; Sundqvist *et al.*, 1998) (Pinheiro *et al.*, 2003a; Pinheiro *et al.*, 2003b). This factor along with its higher prevalence rate, ability to survive within harsh conditions and innate virulence factors have led many researchers to believe that *E. faecalis* plays a major role in the aetiology of persistent/secondary apical periodontitis (Rôças & Siqueira, 2012).

The central role of *E. faecalis* is being seriously questioned by studies using PCR based techniques. Rôças & Siqueira (2012) recently showed that *E. faecalis* may not be the main

pathogen involved in causing post treatment apical periodontitis and that other species such as *Propionibacterium* species, *Fusobacterium nucleatum* (*F. nucleatum*), streptococci, and *Pseudoramibacter alactolyticus* may also be candidate pathogens. Using qPCR, they found that streptococci were present in approximately 40% of cases and comprised 65% of the total bacterial count whereas *E. faecalis* was present in 38% of cases and made up only about 10% of the total bacteria count.

1.1.6.1.2 Survival and virulence factors

E. faecalis contains innate characteristics that help it survive the harsh conditions of the obturated root canal system. *E. faecalis* binds to dentine using a serine protease, gelatinase, and collagen-binding protein (Ace) (Hubble *et al.,* 2003) and is also small enough to invade the dentine tubules to depths which may evade both chemomechanical procedures and intraradicular medicaments. Although it is susceptible to high pH environments such as those produced when in direct contact with calcium hydroxide (Plutzer, 2009) it may survive if the high pH is not maintained (Stenhouse, 2011). *E. faecalis* is also able to exist within a viable non culturable state for extended periods of time and recover by utilising serum and nutritional sources (Figdor *et al.*, 2003). *E. faecalis* is able to form both axenic and multi-species biofilms which helps it resist endodontic treatment (Christo, 2012; Seet *et al.*, 2012; Yap *et al.*, 2014).

E. faecalis possesses certain virulence factors which may help it survive host defences and competition with other microbiota (Sava *et al.*, 2010). Virulence factors include, surface adhesins, cytolysin, aggregation substance, lipoteichoic acid, sex pheromones, hyaluronidase, and lytic enzyme acids (Kayaoglu & Ørstavik, 2004). In addition to these, it is also able to share its virulence traits with other species which may contribute to its survival and disease persistence (Kayaoglu & Ørstavik, 2004). *E. faecalis* is able to suppress the action of lymphocytes (Lee *et al.*, 2004b) adherence to host cells, and also alter the host immune response (Rôças *et al.*, 2004b).

1.1.6.2 *Streptococcus sanguinis*

Streptococcus sanguinis (*S. sanguinis*) (formerly known as "*S. sanguis*") is an indigenous Gram-positive, facultative anaerobic cocci found commonly in the oral cavity

of humans (Socransky *et al.*, 1977). Although *S. sanguinis* forms part of the normal oral flora, this bacterium is an extremely virulent bacteria capable of causing and sustaining severe debilitating diseases such as infective endocarditis (Do *et al.*, 2011) or meningitis (Fukushima *et al.*, 2012). Streptococcus species have been identified as significant opportunistic colonisers of the root canal system in teeth immediately after root canal treatment (Byström & Sundqvist, 1985; Chávez de Paz *et al.*, 2004; Sakamoto *et al.*, 2007) and are particularly prevalent in persistent/secondary apical periodontitis (Molander *et al.*, 1998; Rôças *et al.*, 2004a; Rôças & Siqueira, 2012). A recent study showed that *S. sanguinis* was as prevalent as *E. faecalis*, being found in approximately 30% of teeth with persistent root canal infections (Murad *et al.*, 2014).

S. sanguinis is able to attach firmly to dental hard tissues (Liu *et al.*, 1990) and form both axenic (Bryce *et al.*, 2009) and mixed species biofilms (Yap *et al.*, 2014). Streptococcus species play a central role in the initiation of biofilm formation in the oral cavity (Nyvad & Kilian, 1987; Kolenbrander *et al.*, 2010). Streptococci form coaggregations with other early colonisers such as *F. nucleatum* (Lancy *et al.*, 1983) and together provide a framework on which late colonisers are able to strongly attach and thereby form complex multi-species biofilms (Love, 2012). Streptococci may modulate biofilm formation by producing a range of extracellular factors that either enable metabolic cooperation or antagonism. They may also enhance the growth and virulence of other pathogenic bacteria (Wright *et al.*, 2013).

1.1.6.3 *Fusobacterium nucleatum*

F. nucleatum is an anaerobic, Gram-negative bacterium which is fusiform in shape (Bolstad *et al.*, 1996). It is a common inhabitant of the oral cavity and other areas of the oropharyngeal and gastrointestinal tract (Mira-Pascual *et al.*, 2014). Although found in healthy sites it is a putative periodontal pathogen that is frequently isolated from diseased periodontal sites (Moon *et al.*, 2014) and is an opportunistic pathogen when it spreads from its normal commensal site (Storm *et al.*, 2013). *F. nucleatum* is often associated with primary (Jacinto *et al.*, 2008; Rôças *et al.*, 2011) and persistent/secondary apical periodontitis (Blome *et al.*, 2008; Fujii *et al.*, 2009; Schirrmeister *et al.*, 2009; Rôças & Siqueira, 2012).

F. nucleatum is able to form axenic biofilms (Chew *et al.*, 2012) and multi-species biofilms (Yap *et al.*, 2014). *F. nucleatum* is an important bridging bacteria in biofilm formation as it may coaggregate with both Gram-positive early colonisers and 'late' Gram-negative colonising bacteria. *F. nucleatum* is essential for the coaggregation of many species of bacteria, for example without *F. nucleatum*, the obligate anaerobe *Porphyromonas gingivalis* would not be able to tolerate oxygen tolerant species such as *Streptococcus oralis* (Bradshaw et al., 1998). *F. nucleatum* interacts synergistically with other bacteria to increase population cell density (Sharma *et al.*, 2005) and pathogenicity (Settem *et al.*, 2012). It is also able to modulate the host inflammatory responses (Polak *et al.*, 2009) and adhere to lymphocytes and induce apoptosis (Abe, 2012).

1.1.7 Biofilms

Biofilms are microbial communities that are attached to a surface and encased in an extracellular matrix of polysaccharides and proteins that are of microbial origin (Dufour *et al.*, 2012). Biofilms of the oral cavity are among the most complex human microbiota (Kolenbrander *et al.*, 2010). Before the concept of the biofilm emerged, many studies assumed microbes lead solitary, asocial existences (Nadell *et al.*, 2008). Over time, a paradigm shift occurred as research showed that dense biofilm populations interact extensively with each other, acting more as a whole community for growth and survival, rather than acting as a multitude of singular cells with different characteristics.

Nair (1987) was the first to describe the concept of a biofilm in endodontics. Endodontic biofilms associated with apical periodontitis may be divided into intraradicular, external root (cementum) and periapical biofilms although intraradicular biofilms are by far the most prevalent (Siqueira *et al.*, 2012). Early observational studies have shown that bacteria form dense colonies on the canal walls as well as on intratubular dentine (Sen *et al.*, 1995). Many studies have shown that bacterial biofilms are very prevalent in teeth with primary or persistent/secondary apical periodontitis. The structure of the bacterial community and its proximity and interaction with the host tissues shows that apical periodontitis is a disease primarily caused and sustained by biofilms (Siqueira *et al.*, 2012).

An ideological paradigm shift has occurred in our understanding of how biofilms

primarily cause and sustain apical periodontitis, but despite this, endodontic treatment approaches have not changed considerably within the same time frame. Nair *et al.* (2005) demonstrated that biofilms were not removed from isthmuses and accessory canals of lower molars with apical periodontitis using a single visit treatment philosophy. Unfortunately, inclusion of an inter appointment medicament does not guarantee biofilm removal, especially in areas of the root canal system that are physically removed from the main canal (Vera *et al.*, 2012). Hopefully, as our understanding of biofilms increase, more effective treatment strategies will be developed.

1.1.7.1 Biofilm formation

Biofilms develop on surfaces in a dynamic process which requires a stepwise progression. The first step requires planktonic bacteria to be present and to come in contact with the substrate surface. The substrate surface is often coated with a conditioning film which enhances the initial weak adhesion of bacteria after which stronger adhesions may form (Donlan & Costerton, 2002). Micro colonies then develop from the primary colonisers before covering the surface with a layer of cells. Co-aggregation reactions occur between proliferating bacteria and other planktonic bacteria which increases the density and complexity of the developing biofilm. Various bacteria have different functions which support the growth of the biofilm into macrocolonies. For example, *F. nucleatum* is a bridging organism that allows non-binding bacteria to become a part of the expanding biofilm (Kolenbrander *et al.*, 2010). This process is coordinated through quorum sensing and metabolic communication pathways. Genetic transfer, symbiotic nutritional support along with competitive interactions all shape the membership of the complex biofilm community which increases the efficient utilisation of nutrients while reducing the susceptibility to host defences and therapeutic measures (Love, 2012).

1.1.7.2 The increased protection of bacteria in a biofilm

Bacteria embedded in a mature biofilm are significantly more resistant to antimicrobial agents compared to bacteria in planktonic form (Huang *et al.*, 2011). This however is dependent on many different factors. Biofilms have inherent properties and intricate processes that act to protect the bacterial community which are additives to the inherent defensive mechanisms that individual bacteria possess. The extracellular matrix of the

biofilm provides a physical barrier to the penetration of antimicrobial agents (Branda *et al.*, 2005). Antibiotic diffusion into the deeper portions of the polysaccharide matrix is significantly limited, thereby killing only the surface bacteria and not those inhabiting the lower regions of the biofilm (Dufour *et al.*, 2012). Early colonisers are therefore protected which may then allow the biofilm to re-establish.

Individual bacteria are also phenotypically different when growing as a biofilm structure compared to their planktonic counterparts. Distinct phenotypic sub-populations may arise as a result of bacterial cell responses to the local microenvironment which may help protect the biofilm community (Dufour *et al.*, 2012). It is also interesting to note that axenic biofilms can exhibit different phenotypes even though they can be spatially separated by as little as 10 µm (Socransky & Haffajee, 2002).

Many other phenotypic changes occur within the biofilm that increases the biofilms resistance to antimicrobial agents. For example, growth rate of bacteria in the deeper layers of the biofilm are significantly suppressed after the biofilm has reached a certain structure and complexity level (Donlan & Costerton, 2002). This decreased growth rate protects the microorganisms against antibacterial agents that are more effective against bacteria that are growing at or near exponential growth compared to those in the stationary phase (Portenier *et al.*, 2005). Certain species of bacteria that are nutrient deprived and therefore have a slow generation time have been shown to form biofilms that are more resistant to endodontic irrigating solutions (Liu *et al.*, 2010). Persistent apical periodontitis may also be related to dormant persister cells as these types of cells are able to survive even high concentrations of antibiotics, however the molecular basis for this transition has not yet been clearly elucidated (Dufour *et al.*, 2012). Sedgley *et al.* (2005a) showed that *E. faecalis* could survive in *ex-vivo* obturated canals for greater than 12 months even though all nutrient sources external to the root canal system were removed.

Bacteria communicate within the biofilm via the synthesis of chemicals known as autoinducers which direct a signalling process called quorum sensing. Cell-cell communication may occur between the same or different species using different auto inducer chemicals (Love, 2012). Population density and biofilm formation is regulated by quorum sensing (Davies *et al.*, 1998). Gene expression in response to the auto inducers ultimately affects the phenotypic expression of individual cells, subpopulations and the

biofilm as a whole (Ng & Bassler, 2009). Quorum sensing may also affect a number of other functions in the biofilm such as metabolic functions (Hojo *et al.*, 2009), the release of virulent factors (Pecharki *et al.*, 2008) and dispersion of the biofilm (Solano *et al.*, 2014).

Transfer of genetic information may occur between bacteria via conjugation and thereby increase the virulence of individual bacteria and thus the community as a whole (Kolenbrander *et al.*, 2010). Environmental pressures may then favour the growth of more virulent subpopulations of cells, thereby enabling the biofilm to adapt to environmental pressures and resist elimination (Chávez de Paz, 2007).

1.2 Endodontic treatment

The aim of endodontic treatment is to maintain the health of all parts of the dental pulp and the associated periapical tissues. When this is not possible, the aims are to remove the disease-causing agents, and prevent the re-establishment of disease. To accomplish these aims, multiple approaches are used including: mechanical instrumentation; irrigation using an antimicrobial and tissue dissolving agent; the judicious use of intraradicular medicaments between appointments; obturation of the root canal space and restoration of the coronal portion of the tooth to prevent reinfection.

Chemomechanical preparation of the root canal system combines the two elements of instrumentation and irrigation. Instrumentation physically aids in the removal of bacteria and organic tissue (vital and necrotic) from the root canal and tubules that are immediately surrounding the canal walls. In addition, canal preparation allows irrigants and antibacterial medicaments to be placed deep within the root canal system, thereby facilitating penetration to areas that are inaccessible to endodontic instruments. The efficacy of mechanical debridement directly impacts the efficacy of both the antibacterial agents and the obturation of the canal.

1.2.1 Mechanical instrumentation

Mechanical instrumentation aids in the cleaning and shaping of the main root canal (Schilder, 1974). The classic study by Byström & Sundqvist (1981) showed that mechanical instrumentation using saline irrigation was able to achieve a 100-1000 fold

decrease in bacteria numbers, however all canals still contained bacteria at the end of the first visit. Only with the use of an antibacterial irrigating solution such as sodium hypochlorite (NaOCl) could further reductions in bacteria be predictably achieved in one visit (Byström & Sundqvist, 1983).

Mechanical instrumentation of the apical region to large sizes may lead to further reductions in microbial load. Ørstavik *et al.* (1991) sampled bacteria from single rooted teeth with apical periodontitis that were mechanically instrumented to various sizes using saline as the irrigant. They found a higher number of canals contained bacteria when prepared to an apical file size of ISO 35 or 40 compared to canals prepared to large sizes. Dalton *et al.* (1998) confirmed these results using lower molar mesial roots.

Mechanical instrumentation physically disrupts a significant proportion of the biofilm while also removing the most infected layer of dentine that surrounds the main canal walls. Dalton *et al.* (1998) showed that an initial rapid rate of bacterial reduction was achieved by the first files that removed the most infected layers of dentine; however this effect was reduced with each subsequent increase in apical size. In other words, increasing the apical preparation size does not lead to a linear reduction in bacteria number.

Bacteria penetrate dentine tubules to deeper levels in the coronal and middle thirds than in the apical part of the canal (Love, 1996; Matsuo *et al.*, 2003). It is reasonable to assume that more bacteria will be removed from the root canal if the taper of the preparation is increased. This however has not been clearly shown. Aydin *et al.* (2007) infected premolar teeth with *E. faecalis* for seven days before instrumenting the canals using two different rotary NiTi instrument systems with varying tapers. The apical preparation size (ISO 30) was the same for each group and the teeth were irrigated with distilled water using a syringe. Significant bacteria reductions were achieved with both tapers however even though the larger taper removed approximately 60% more dentine than the smaller taper, no significant difference was seen between the two groups. This is most likely because both tapers sizes were large enough to facilitate the effective removal of loose biofilm and dentine when the apical sizes were the same. It must be noted that, paper points were used to sample bacteria after the canals were scraped with a size 15 Hedstrom file. Given that both techniques reduce bacteria levels by over 97%, this technique may not be sensitive enough to sample enough disrupted biofilm or infected dentine to be able to show

difference between the groups.

Most rotary endodontic NiTi files have a different cutting action and preparation procedure compared to traditional hand filing with stainless steel instruments (Rôças *et al.*, 2013). Dalton *et al.* (1998) showed that similar levels of bacteria reduction could be achieved with both the rotary NiTi instrumentation technique and standard step-back technique using stainless steel hand K-files when saline was used as the irrigant. Rôças *et al.* (2013) confirmed these results using different tapered hand and rotary instruments. Both of these studies found that instrumentation was not effective at completely removing bacteria from the main root canal. Micro-CT imaging has shown that mechanical instruments do not come in contact with all surfaces of the root canal walls (Peters *et al.*, 2001). Paqué *et al.* (2010) found that approximately 65-75% of the apical 4 mm remained untouched when either rotary or hand instruments were used to mechanically prepare oval shaped distal root canals of lower molars. These studies demonstrate the inadequacy of mechanical preparation alone to reduce bacteria levels that are most likely necessary for periapical health (Sjögren *et al.*, 1997). They also highlight the need to use an antibacterial solution to improve the efficacy of the cleaning and shaping of infected root canals (Shuping *et al.*, 2000).

Maximising the mechanical removal of bacteria from the root canal system is achieved by increasing both the apical size and canal taper. No two infected root canal systems are identical, as each canal varies in length, volume, anatomical complexity and total bacterial load. Clinically, an intelligent decision needs to be made about the extent to which an individual canal should be prepared in order that the overall aims of endodontic treatment are achieved while minimising the chance for iatrogenic damage.

1.2.1.1 The limitations of instrumentation due to the root canal anatomy

The root canal system contains many complex anatomical features such as isthmuses, ramifications, deltas, accessory and lateral canals (Vertucci, 1984). The pulpal space may also become calcified as a result of the natural ageing process (Burke & Samarawickrama, 1995) or as a response to low-grade bacterial infection or trauma (Hermann *et al.*, 2012). Internal or external resorption (Vier & Figueiredo, 2004; Heithersay, 2007; Rossi-Fedele *et al.*, 2010) may also occur as a result of trauma, infection, systemic disease (Armas *et al.*,

2008) or other causes. Natural variation between teeth types (Vertucci, 1984) and developmental anomalies such as dens invaginatus (Brooks & Ribera, 2014) may increase the complexity of the root canal system and produce significant challenges to removing bacteria from the root canal system using currently available methods. Many different studies have shown that complete mechanical instrumentation of the root canal system cannot be realistically achieved (Endal *et al.*, 2011).

Following infection of the main root canal, bacteria are able to penetrate the dentine tubules to varying depths. The depth of penetration may be affected by the type of bacteria, diameter and contents of the dentine tubules (Harrison *et al.*, 2010; Ran *et al.*, 2014). Mechanical debridement does not effectively remove bacteria from the tubules. Matsuo *et al.* (2003) showed that bacteria invaded dentine tubules in 70% of teeth diagnosed with apical periodontitis. Mechanical preparation of the canal reduced the frequency of invasion by only 5%. This highlights the need for other modalities to be employed to remove bacteria from the root canal system.

The lower molar mesial root canal system is considerably more complex than roots with a single canal. Most lower molar mesial roots have two canals, however occasionally there are three or more canals present. Approximately 40% of lower molar mesial roots have two canals that join before the apex and thereby share a common foramen whereas the other 60% generally have two separate canals with separate foramen (Vertucci, 1984). In addition to the presence of canals, histological studies and micro-CT studies show that the root canal morphology may be incredibly complex with webs, fins, anastomoses and isthmus regions that are impossible to instrument using currently available technology.

Vertucci (2005) described an isthmus as a “narrow, ribbon-shaped communication between two root canals that contains pulp or pulpally derived tissue”. Once bacteria enter the root canal system, an isthmus may function as a bacterial reservoir. A study by Fan *et al.* (2010) using micro-computed tomography showed that 85% of mandibular first molars had isthmus lengths of approximately 2.5 mm that were located within 5 mm of the apical foramen (Fan *et al.*, 2010). It has also been shown that an isthmus may extend the entire length of the canal (Endal *et al.*, 2011). Paqué *et al.* (2011) showed that mechanical preparation of the lower molar mesial root canals caused considerable amounts of dentine to be forced into the isthmus region. Endal *et al.* (2011) repeated the experiment using

copious amounts of irrigant during the instrumentation procedures to determine whether this would make a difference. Despite using an irrigant they found that approximately 35% of the isthmus space was filled with debris after treatment. Removing all infected debris and biofilm from the isthmus region is a clinical dilemma that currently has no solution. It is therefore important to try and maximise infected debris removal by using a combination of irrigants and an inter-appointment medicament so that the total microbial load will be low enough to be conducive with periapical health (Siqueira & Rôças, 2011).

1.2.2 Irrigation

Many different solutions have been used to irrigate the root canal system during endodontic treatment. The ideal irrigant should have a number of properties: a) broad antimicrobial spectrum b) dissolve organic substances such as pulp tissue and biofilms c) inactivate endotoxins d) prevent the formation of a smear layer or at least dissolve it once it has formed e) be minimally toxic to vital periodontal tissues and f) have low allergenic potential (Zehnder, 2006). Unfortunately a single irrigating solution is unable to cover all these areas, therefore a combination of solutions needs to be used to attain the desired outcomes while avoiding the possibility of adverse outcomes.

Because of their complimentary properties and superior outcomes, the most commonly used irrigating solutions are NaOCl and EDTA (ethylene diamine tetra-acetic acid). Other solutions that are currently used in endodontic practice include chlorhexidine, MTAD (a mixture of tetracycline isomer, acid and detergent), Tetraclean (containing doxycycline hyclate, acid and a detergent), hydrogen peroxide, iodine compounds, alcohol, local anaesthetics and saline. The efficacy of these irrigating solutions may be limited by their inherent properties and also by both the root canal anatomy and the delivery mechanisms.

Sodium hypochlorite has superior antimicrobial properties compared to other endodontic irrigants. Dunavant *et al.* (2006) developed a flow cell to grow *E. faecalis* biofilms on ceramic discs before treating the discs with various irrigating solutions. They found that both 1% and 6% NaOCl were more effective at eradicating bacteria than either Smear Clear™, 2% chlorhexidine, REDTA and BioPure™ MTAD™. The primary reason for this is that apart from NaOCl, all other irrigants were ineffective at dissolving the biofilm. Wang *et al.* (2012) confirmed the superior antimicrobial and biofilm dissolving

properties of NaOCl and also demonstrated its superior ability to remove bacteria from the dentine tubules compared to QMIX and chlorhexidine.

Violich & Chandler (2010) described the smear layer that forms during instrumentation of the root canal as being a layer of organic and inorganic material that may also contain bacteria and their by-products. Removing the smear layer from the root canal walls and dentine tubules is important so that antibacterial solutions have a greater chance of reaching and killing bacteria residing in the dentine tubules. Ethylene diamine tetra-acetic acid or EDTA with cetrimide (EDTAC), have both been used for the last 4 decades in endodontics because of EDTA's excellent chelating properties (Zehnder *et al.*, 2005). It has been shown to be able to remove the smear layer in the coronal, middle and apical third of the roots (Violich & Chandler, 2010). Byström & Sundqvist (1985) showed microbial reduction was improved when 15% EDTA was used as an adjunct to 5% NaOCl. Many studies since have shown that the full potential of antimicrobial irrigants may be realised after the smear layer has been removed (Baumgartner & Mader, 1987; Soares *et al.*, 2010; Neelakantan *et al.*, 2014).

1.2.2.1 Sodium hypochlorite

Sodium hypochlorite is the most commonly used and effective antimicrobial endodontic irrigant (Willershausen *et al.*, 2014). In water, NaOCl forms two highly reactive oxidising agents: hypochlorite ion (OCl^-) and hypochlorous acid (HClO). Hypochlorite ion has a high tissue-dissolving capacity whereas the hypochlorous acid is highly antibacterial. Their concentration in solution is pH dependent. Hypochlorous acid predominates between pH 4 and 7 (pKa = 7.53) whereas when the pH is above 9, hypochlorite ion predominates (McDonnell & Russell, 1999). As an endodontic irrigant, NaOCl has an approximate pH of 12 (Frais *et al.*, 2001) however the pH may vary (pH 10.9 - 12) between manufacturers and this may affect its tissue dissolving capabilities (Clarkson *et al.*, 2006). In its two forms, NaOCl interferes with enzymatic activity, cellular metabolism and phospholipid and protein degradation (Estrela *et al.*, 2002; Winter *et al.*, 2008).

1.2.2.1.1 Tissue dissolving capabilities and antibacterial action

NaOCl has a broad antibacterial action against a variety of Gram-negative, Gram-

positive (Byström & Sundqvist, 1981), and spore-forming microorganisms (Waltimo *et al.*, 1999). It also has the ability to dissolve necrotic and vital tissues such as the dental pulp (Senia *et al.*, 1971; Clarkson *et al.*, 2006). The importance of time, concentration, agitation and volume applies to both the dissolution of biofilm and eradication of bacteria.

The volume of NaOCl in relationship to the organic matter is important because the activity of the NaOCl is rapidly depleted when in contact with a large organic surface area. Smaller volumes of NaOCl will be comparable if the concentration is increased as higher concentrations of NaOCl contain larger reservoirs of chlorine ions that are free to react with organic matter (Clarkson *et al.*, 2013). If NaOCl is stagnant, tissue dissolution will take considerably longer than if mechanically agitated. Mechanical agitation of NaOCl increases the fluid velocity and therefore improves the exchange of reactive ions that are involved in dissolving activities with those that have become inactivated by organic matter (Moorer & Wesselink, 1982). Although not commonly used in clinical practice, the tissue dissolving capacity of NaOCl may be enhanced by heating the solution before use (Cunningham & Balekjian, 1980). This concept has been suggested as an alternative to using higher concentrations of NaOCl and therefore reduce possible toxicity, however this hypothesis has not been readily substantiated and requires further research.

Sodium hypochlorite needs a sufficient amount of time for the chlorine ions to interact with the organic tissue. Spratt *et al.* (2001) using membrane filtered discs showed that the contact time of NaOCl was an important variable in improving the antibacterial action of NaOCl against biofilms. If the contact time is shortened then the concentration of the NaOCl must be increased.

In vivo studies report conflicting results as to whether higher concentrations of NaOCl are more effective than low concentrations. Time, volume, temperature and agitation are other important variables that may either decrease or increase the efficacy of NaOCl. Siqueira *et al.* (2000) did not find a difference between 1%, 2.5% and 5.25% NaOCl after chemomechanical preparation of extracted human single rooted teeth that had been infected with *E. faecalis*. They reasoned that higher concentrations of NaOCl may not be needed if copious and frequent applications of NaOCl at low concentrations occur, such that sufficient chlorine ions are available to eliminate bacteria. However, the infected roots were inoculated and incubated for only 24 hours which is an insufficient time to allow for a

mature biofilm to be established. Stojicic *et al.* (2013) showed using collagen-coated hydroxyapatite discs that a three week old biofilm is significantly more resistant to eradication by NaOCl compared to a 2 week old biofilm. They also showed that 3% NaOCl had a significantly higher kill rate than 1% NaOCl.

Berber *et al.* (2006) grew *E. faecalis* in extracted human premolar teeth for three weeks to establish a mature biofilm before chemomechanically preparing the teeth using hand and rotary instruments and various concentrations of NaOCl. Using paper point sampling methods they found no significant difference in the antibacterial effect in the main canal where the irrigant could easily flow and be replenished. They also sampled bacteria in the dentine tubules that may have survived treatment by taking dentine shavings from the root canal. They found that 5.25% NaOCl had a greater antibacterial activity than 0.5% and 2.5% NaOCl. Increased penetration of sodium hypochlorite into dentine tubules may also be enhanced if activated by either ultrasonic (Harrison *et al.*, 2010) or laser energy (Neelakantan *et al.*, 2014).

Many studies have assessed the efficacy of different concentrations of NaOCl in the laboratory setting. This is in stark contrast to the paucity of *in vivo* studies, especially given the detrimental effects that may occur if NaOCl is extruded through the apex (Zhu *et al.*, 2013b). Cvek *et al.* (1976) and Byström & Sundqvist (1983) both used 0.5% and 5% NaOCl in their treatment of teeth with apical periodontitis. They found no difference between the concentrations with regards to the antibacterial effect achieved. This may be because endodontic treatment was performed on anterior teeth with relatively large canals where large quantities of irrigant can be delivered to most areas of the canal. They also used paper point sampling methods which samples only the canal contents and its walls. It is unknown whether 0.5% will be as effective as 5% NaOCl in reducing bacteria levels in posterior teeth.

1.2.2.2 Irrigation dynamics

Traditionally irrigation of the main root canal has been performed using a needle and syringe under positive pressure (Chang *et al.*, 2014). Regardless of the delivery method, it is essential that an irrigant such as NaOCl be able to penetrate fully into the root canal system and therefore remove microbial biofilms, pulp tissue and dentine debris. There are

however a number of different factors which influence the efficacy of the irrigation protocol such as the canal and needle size, needle depth, canal curvature, flow rate, irrigant volume and the presence of a vapour or fluid lock.

The natural or prepared size (apical preparation and diameter) of a canal is important because it directly impacts the flow of an irrigant. Small sized canals such as those with a diameter of 0.2 mm resist the flow of irrigants more than larger sized canals such as those prepared to a diameter of 0.4 mm wide. Increasing the canal diameter or taper improves the efficacy of irrigation. It is hypothesized by Huang *et al.* (2008) that a larger apical size improves the flushing effect in the apical region and that larger tapered canals allow better irrigant exchange between spent molecules in the apical region and the unreacted NaOCl molecules in the coronal portion of the canal. This is in agreement with Boutsioukis *et al.* (2010b) and Boutsioukis *et al.* (2010a). Falk & Sedgley (2005) using a small 28-gauge endodontic needle found that canals prepared to size 60 (with 0.04 taper) were more effectively irrigated than canals prepared to size 36.

The depth to which a needle can be inserted into a canal is directly related to the size of both the needle and the canal. Traditionally, size 23 or 25 gauge needles were used however smaller gauge needles (size 27 – 30) have been recommended (Gulabivala *et al.*, 2010). This is because when a side-vented needle and syringe irrigation are used in a safe manner in a canal, the irrigant seldom flows 1 mm beyond the tip of the needle (Chow, 1983). This is the reason why the needle tip can be placed in close proximity to the apical foramen so that the apical part of the canal is cleaned effectively (Boutsioukis *et al.*, 2009). Smaller diameter needles 30-gauge have been shown to be more effective than larger sized needles (22-gauge) at cleaning radiopaque contrast medium from the apical third of root canals (Guerreiro-Tanomaru *et al.*, 2013). This finding was the same for all stages of the root canal widening (sizes 20, 30 and 40 K-files with an 0.02 taper) procedure and no difference was found between the 30-gauge needle with either an open-ended or side-vented design. The risk of extrusion however is greater with the open-ended compared to a side-vented needle design (Chang *et al.*, 2014).

The flow rate of an irrigant may play an important role in the cleaning process (Chang *et al.*, 2014). Hydrodynamic shear stress is created when an irrigant flows past a surface (Boutsioukis *et al.*, 2010a). Increasing the flow rate will increase the sheer stress on the

canal walls which may increase debris and biofilm removal. Increasing the flow rate of an irrigant such as NaOCl is not without its risks and so a balance needs to be achieved between maximising irrigant flow while ensuring patient safety (Boutsioukis *et al.*, 2014). Negative apical pressure devices (e.g. the Endovac System) may reduce the risk of extrusion while increasing debris removal (Goode *et al.*, 2013; Khan *et al.*, 2013).

In the *in vivo* environment, the canal behaves as a closed-end channel. An "air lock" or "vapour lock" may form in the apical portion of the canal during irrigation (Tay *et al.*, 2010). This may prevent the flow of irrigant into the apical 1 to 2 mm of the canal: the site closest to the periapical tissues. Tay *et al.* (2010) showed that debridement efficacy is reduced when an apical vapour lock occurs and this may occur even when using a small side-vented (30-gauge Max-i-Probe) needle.

Recently, Boutsioukis *et al.* (2014) showed that an apical vapour lock may occur under certain conditions during syringe irrigation however increasing the apical size (from 35 to 50) and using an open-ended needle resulted in a smaller vapour lock. They demonstrated that an established apical vapour lock could be removed by briefly inserting the needle to working length while irrigating at a low flow rate of 0.083 mL s^{-1} and then moving the needle tip away from the apex for the continuation of irrigation procedure (Boutsioukis *et al.*, 2014). This flow rate is commonly used by cautious practitioners (Boutsioukis *et al.*, 2007) and is a clinically relevant suggestion, however an open-ended needle placed within close proximity to the apical foramen increases the chance of extrusion (Psimma *et al.*, 2013). This may be reduced or avoided with the use of a side-vented needle in canals with small apical foramen size.

To minimise the risk of extrusion but ensure that the apical 1 to 2 mm is well irrigated it seems prudent to use a side-vented needle positioned within 1 mm of the working length. An open-ended needle tip should be positioned no closer than 2 mm from the working length. In both situations the needle should not bind in the canal and a light to moderate flow rate should be used (Boutsioukis *et al.*, 2009; Boutsioukis *et al.*, 2014). Consideration of key anatomical structures in close proximity to the root apex such as the sinus should be taken into consideration when making a clinical decision about needle tip design, placement and flow rate. It may be wiser in some circumstances to use alternative devices such as ultrasonic or sonic energy to improve irrigant dispersal.

The volume of irrigant that is used during chemomechanical preparation is important as it may affect the cleaning efficacy. For example, Sedgley *et al.* (2005b) irrigated canals with a 28-gauge (Max-I-Probe) needle at different positions (1 or 5 mm from the working length) within the canal. They showed that significantly more bioluminescent bacteria were removed from the canal when the volume of the irrigant was increased. This study also confirmed that positioning the needle tip closer to the working length also improved reductions in bacterial load.

Root curvature directly impacts the flow of irrigant into the apical third of the canal by impeding the flow of irrigant. Nguy & Sedgley (2006) instrumented premolar root canals to apical sizes of 27, 36 and 46 with rotary instruments (0.04 taper) before inoculating the canal with bioluminescent bacteria. They then irrigated the canals using a 30-gauge needle positioned 1 mm away from the working length. They found that irrigation was significantly less effective in moderately curved roots and that irrigation efficacy could be improved by increasing the apical preparation.

Bronnec *et al.* (2010) showed that improved irrigation is achieved in curved canals by increasing the apical size, canal taper and irrigant volume while ensuring the needle is positioned within close proximity to the apex. This study showed that the principles affecting irrigating efficacy in straight canals are similar to those governing curved canals however it may not be possible to change the variables to the same extent in curved canals because increasing the canal taper and apical size may cause iatrogenic damage.

1.3 Ultrasonic activated irrigation

An ultrasonic device transforms electrical energy into ultrasonic waves of a particular frequency by either magnetorestriction or piezoelectricity. In dentistry the piezoelectric device is the most popular (Park, 2013) and the frequency is fixed at 30 kHz (van der Sluis *et al.*, 2007). Although introduced into endodontics in 1957, ultrasonic devices gained significant popularity in the 1980s for mechanical preparation of the root canal (Martin, 1976). After about a decade of use, their use declined as it proved difficult to control the cutting action of dentine and iatrogenic damage could easily occur (Lumley *et al.*, 1992). In 1976, Martin showed that ultrasonics could increase the efficacy of sodium hypochlorite

against bacteria in a root canal (Martin, 1976). This paved the way for ultrasonics to be investigated more thoroughly as an adjunct to the traditional irrigating protocols.

The passive ultrasonic irrigation concept was first described by Weller *et al.* (1980) and describes the process by which acoustic energy is transmitted via a non-cutting wire to an irrigant in the root canal (van der Sluis *et al.,* 2007). The term "passive" does not indicate that the file is not active within the irrigant but rather that it is not "active" in cutting tooth structure. The term "ultrasonically activated irrigation" (UAI) has been mentioned by others (Harrison *et al.*, 2010) although only recently has it been advocated as a better term to use than passive ultrasonic irrigation as it more accurately describes this method of irrigation while minimising confusion (Boutsioukis *et al.,* 2013). This term has not yet gained widespread popularity.

1.3.1 Acoustic microstreaming and acoustic cavitation

The ultrasonic waves created by acoustic energy are able to induce acoustic streaming of an irrigant (Ahmad *et al.*, 1987). Acoustic streaming is the swift movement of fluid in a vortex-like or circular motion around a vibrating file (Walmsley *et al.*, 1989). Within the confines of the root canal, acoustic microstreaming arises from the frictional forces between a boundary and a medium carrying vibrations of circular frequency (van der Sluis *et al.*, 2007). Rapidly moving eddy currents occur around the file in distinctive patterns that corresponds to the nodes and antinodes along the length of the file. While noting the occurrence of primary and secondary flow patterns, Ahmad *et al.* (1987) found that fluid moved from the apical end of the file towards the coronal aspect. It was also noted that fluid circulated with the greatest velocity and strength around the tip of the file compared to the coronal area of the file. Within the root canal, this pattern of flow may facilitate the cleaning process by moving debris contained in the apical portion towards the coronal portion of the canal (van der Sluis *et al.*, 2007).

Acoustic cavitation and acoustic microstreaming have been claimed as the mechanisms by which UAI may improve the cleaning efficacy of a root canal (Jiang *et al.*, 2012; Boutsioukis *et al.,* 2013). Acoustic cavitation is the creation of new bubbles or the expansion, contraction and/or distortion of pre-existing bubbles in a liquid. The expansion and rapid collapse of a bubble produces a focus of energy leading to intense radiating

shockwaves (van der Sluis *et al.*, 2007). Stable cavitation is reported to produce more gentle volumetric oscillations whereas transient cavitation is of main interest because it produces violent inertial collapse of a bubble that may enhance chemical reactions (Tiong & Price, 2012) and surface cleaning (Fernandez Rivas *et al.*, 2012). Only recently has it been shown using sonochemiluminescence that transient cavitation does occur within the root canal confines when using an ultrasonically driven file (Irrisafe) that is set at clinically relevant power setting (Macedo *et al.*, 2014a). It has also been recently proven that cavitation and microstreaming may still occur even when the file unintentionally touches the canal wall, although the intensity is reduced (Boutsioukis *et al.*, 2013).

1.3.2 Dissolution of organic matter

Ultrasonic activated irrigation improves tissue dissolution (Moorer & Wesselink, 1982) in NaOCl. The main reason for this is because acoustic cavitation and microstreaming causes increased fluid movement which improves the reaction rate (Macedo *et al.*, 2010) between active chlorine molecules and organic matter such as dentine or pulp tissue remnants. Ultrasonic activation may increase the temperature of the intraradicular irrigant by up to 10°C however a temperature rise of this amount does not lead to significant improvements in the reaction rate (Macedo *et al.*, 2014c).

1.3.3 Removal of debris

Removal of both organic and inorganic debris from the root canal and its irregularities is considered important because the debris may contain viable microorganisms, limit the effectiveness and penetration of intraradicular medicaments and also affect the obturation of the root canal space (Siqueira *et al.*, 1999). Many different studies using a variety of models have compared the effectiveness of UAI and conventional syringe irrigation in removing debris from the apical third of the canal and un-instrumentable recesses (van der Sluis *et al.*, 2007). The general consensus is that ultrasonic activated irrigation improves debris removal (Plotino *et al.*, 2007; Jiang *et al.*, 2012) however not all studies have found a difference between the irrigating methods (van der Sluis *et al.*, 2007).

Removal of debris from posterior teeth with complex root canal systems is a major challenge. Ultrasonic activated irrigation has been shown to improve debris removal from

the isthmus region compared to syringe irrigation (Burleson *et al.*, 2007; Thomas *et al.*, 2014). The reason for this may be because acoustic microstreaming generates hydrodynamic shear stresses on the root canal walls. The intensity of the hydrodynamic shear stress is proportional to the streaming velocity, which is at its maximum at the tip of the vibrating file (Roy *et al.*, 1994). A recent computational fluid dynamics study by Chen *et al.* (2014) showed that UAI produced the highest shear stress along the root canal wall compared to open and closed needles delivered by a syringe or a negative apical pressure irrigating device. The high shear stresses and lateral movement of the fluid may improve the interaction of irrigant with intraradicular debris and most likely biofilm however little is known about how the intraradicular biofilm is disturbed by ultrasonic devices. Macedo *et al.* (2014b) recently showed that NaOCl combined with UAI improved the removal of a biofilm-mimicking hydrogel from the isthmi region and lateral canals compared to syringe irrigation. Studies are yet to be done on how UAI may remove biofilm from the isthmus region as this is far more important than merely investigating debris removal.

1.3.4 Curved canals

Ultrasonic activated irrigation can be effective in removing debris from curved canals (Gutarts *et al.*, 2005; Malki *et al.*, 2012) however the best results are achieved when the file tip is pre-bent (Lumley *et al.*, 1992). Pre-bending the file tip minimises contact between the file and canal wall. Acoustic streaming and cavitation are more effective when the file does not contact the canal wall however light contact does not entirely stop the hydrodynamic phenomenon (Boutsioukis *et al.*, 2014). UAI may perform better than syringe irrigation in curved canals with isthmi (Gutarts *et al.*, 2005) (Al-Ali *et al.*, 2012) however this is not always the case (Amato *et al.*, 2011).

UAI is maybe more effective than syringe irrigation in curved canals because UAI improves the flow of irrigant to within close proximity of the desired working length (Merino *et al.*, 2013). An *in-vivo* study by Munoz & Camacho-Cuadra (2012) found that conventional irrigation with a 27-gauge needle was ineffective in delivering irrigant to within 1 mm of the working length in mesial canals of mandibular molars with moderate to severely curved roots even though the needle tip was placed within 2 mm of working length. In comparison UAI consistently caused the irrigant to flow to within 0.5 mm of the working length. An *in vitro* study by Malki *et al.* (2012) showed that when the ultrasonic

file tip was placed without constraint in a curved root canal (just before the curve) the irrigant flowed approximately 3 mm in front of the file tip and improved debris removal compared to syringe irrigation. The cleaning effect reduced as the distance increased from the tip of the ultrasonic file. Irrigant penetration may be improved if the ultrasonic intensity is increased (Jiang *et al.,* 2011). These studies show that UAI may be useful in improving the flow of irrigant into the apical third and therefore improve debris removal.

1.3.5 Smear layer removal

A smear layer is produced on root canal walls during mechanical preparation. To remove the smear layer using syringe irrigation, a final rinse with EDTA followed by NaOCl has been shown to be an effective combination (Baumgartner & Mader, 1987). Ultrasonic agitation of high concentrations of NaOCl for extended periods of time may lead to the removal of the smear layer in some areas of the canal (Alacam, 1987). Other studies have shown that ultrasonication of NaOCl is ineffective (Ahmetoglu *et al.,* 2014) and does not enhance the removal of the smear layer when EDTA is incorporated into the irrigating protocol (Abbott *et al.*, 1991).

Removing the smear layer from the apical third is less predictable than removing it from the middle and coronal thirds of the root canal (Andrabi *et al.*, 2014). Mancini *et al.* (2013) recently showed that UAI was more effective than continuous syringe irrigation in removing the smear layer in the apical third when both EDTA and NaOCl were used in sequence as the final rinsing protocol. This study clearly showed that EDTA may be more effective in the apical third if its penetration is increased by the use of ultrasonic agitation.

1.3.6 Biofilm removal

The physical mechanisms by which acoustic microstreaming and cavitation may disrupt intraradicular biofilm are yet to be elucidated. Before 2010, the antibacterial effects of UAI were focused on removing planktonic bacteria from the main root canal through a flushing effect (van der Sluis *et al.*, 2007). Only recently has the efficacy of UAI begun to be investigated using biofilm models.

Bhuva *et al.* (2010) was the first study to test the efficacy of ultrasonic activated

irrigation in removing an *E. faecalis* biofilm from extracted single-rooted human teeth. They mechanically prepared the roots to size 50 with a taper of 0.10 so that the ultrasonic file could oscillate without impediment. The biofilm was grown for 72 hours before being treated using UAI or syringe irrigation and 1% NaOCl. For the ultrasonic group, a total of 4 mL of irrigant was used with two 20 second cycles of ultrasonic activation whereas 6 mL of irrigant was used for the syringe irrigation group. SEM analysis showed no difference between UAI and syringe irrigation with regards to biofilm removal. Although this study was well standardised and calibrated, it lacks clinical significance because the *E. faecalis* biofilm was only grown for 72 hours before being treated. The apical preparation size, canal taper and irrigant volume were all sufficiently large enough to enable NaOCl to come in contact with almost all areas of the canal, therefore it is not surprising to find that both groups removed over 95% of the biofilm layer in the areas examined. UAI appears to have no major benefit over syringe irrigation in removing immature biofilm when the root canal dimensions are suitable for adequate irrigant penetration using conventional syringe irrigation methods.

A number of studies have reported similar findings to Bhuva *et al.* (2010) using a mature biofilm model. Gründling *et al.* (2011) using SEM analysis found no difference between UAI and syringe irrigation in removing 50 day old *E. faecalis* biofilm from bovine root canals that were prepared to an apical size of 60. Peters *et al.* (2011) measured bacteria reduction as assessed by paper point sampling and viability counting (CFU/mL). They found no difference between the two treatment modalities with regards to mean bacteria reduction and the number of samples which showed negative growth. Both UAI and syringe irrigation were ineffective at predictably eliminating bacteria from the canal walls. They used paper points to sample the canal contents and found that less than 10% of the canals were free from bacteria. The mean and median bacteria levels were in the order of 10^6 CFU/mL. Irrigating with 6% NaOCl for longer than 60 seconds may have removed more biofilm and made their findings more clinically relevant.

Alves *et al.* (2011) grew *E. faecalis* in premolar roots for 30 days before treating the teeth using traditional chemomechanical procedures. They found that 9 of 20 samples returned negative cultures. One minute of ultrasonic activation using 2.5% NaOCl increased the number of negative cultures to 13, a final rinse with chlorhexidine, increased the number of negative cultures to 16 roots. Only by using both UAI and a final rinse with

chlorhexidine solution was a statistically significant increase in negative samples found in comparison to conventional measures. The number of teeth used in this study was small and no power study was performed. Their results suggest that if a larger sample size was used they may have found a statistical difference between UAI and syringe irrigation.

Ultrasonic activated irrigation may improve the removal of bacteria from the dentine tubules and may improve removal of mature biofilm from the canal walls. Harrison *et al.* (2010) established a 4 week *E. faecalis* biofilm in straight roots before chemomechanically preparing the single canals (apical size 30, 0.09 taper) using 1% NaOCl. The final rinsing protocol included the use of 15% EDTA to remove the smear layer followed by a final flush of 1% NaOCl. One third of the roots received no further treatment; another third received an additional 1 min of ultrasonically activated irrigation with a continuous flow of 1% NaOCl. In the remaining roots, calcium hydroxide medicament was placed in the canals and removed after seven days. Analysis using SEM and light microscopy showed that bacteria were not eliminated from the canal walls in roots that received no adjunctive treatment, whereas the root canal walls were free from bacteria in both the ultrasonic and calcium hydroxide groups. Bacteria invaded tubules to an approximate depth of 150 µm. In the roots that had only received routine cleaning, bacteria in tubules remained in most segments of the root. Only a small number of segments contained detectable bacteria in the ultrasonic and calcium hydroxide groups and no difference was found between these groups. Within the limitations of this study, UAI may be an effective supplementary means of reducing microbial load in dentine tubules in comparison to cleaning and shaping alone.

A recent study by Neelakantan *et al.* (2014) does not support the findings of Harrison *et al.* (2010). Neelakantan *et al.* (2014) did not find a difference between the efficacy of UAI and syringe irrigation using 3% NaOCl to remove a mature four week old *E. faecalis* biofilm from the root canal wall and dentine tubules to a depth of 400 µm. The canals in this study were irrigated with 3% NaOCl for a total of four minutes. The needle tip was positioned 1 mm away from the working length. These irrigating parameters are ideal for ensuring fresh NaOCl comes in maximum contact with the biofilm layer lining the canal wall. Therefore the beneficial hydrodynamic and chemical efficacy that is produced by ultrasonic activation of NaOCl (Macedo *et al.*, 2010) (Macedo *et al.*, 2014b) may have negligible additive effect if NaOCl is able to be delivered to all areas of the canal using a syringe with adequate volume. These experiments need to be repeated in teeth with

complex root canal systems that hinder the removal of biofilm using conventional chemomechanical measures.

1.4 Laser activated irrigation

The first functioning laser was developed in 1960 (Maiman, 1960). A decade later it was used in endodontic research to attempt to seal the apical foramen (Weichman & Johnson, 1971). Since that time lasers have been used for a variety of endodontic purposes such as dental pulp vitality testing, vital pulp therapy, pulpotomy procedures, dentinal hypersensitivity, shaping of the root canal, disinfection of the root canal system, obturation, peri-radicular surgery and tooth bleaching (Mohammadi, 2009). Disinfection of the root canal system using lasers has been attempted using either direct irradiation of the root canal wall (Wang *et al.*, 2007), photo activated disinfection (Chrepa *et al.*, 2014) and laser activated irrigation (George & Walsh, 2008).

1.4.1 Laser activated irrigation definitions

Laser Activated Irrigation (LAI) is the general term used to describe the irradiation of an irrigant within a root canal (George & Walsh, 2008). The word “activation” is used to primarily describe the creation of physical turbulence as a result of laser energy. Although somewhat confusing, the word activation does not indicate that a previously inactive irrigant has been made chemically active by the laser. The laser does however increase the chemical efficiency and efficacy of certain endodontic solutions. For example, Macedo *et al.* (2010) showed that laser activation of NaOCl in bovine teeth significantly increased the consumption of freely available chlorine in solution compared to a NaOCl that received no activation. The most likely reason for this is the rapid increased movement of the molecules (convection) in the root canal system caused by cavitation. The increased reaction rate of NaOCl was also found during rest intervals when the NaOCl was not being activated however the mechanisms of this phenomenon are not understood (Macedo *et al.*, 2010).

A recent protocol for LAI has been developed and marketed as Photon-Initiated Photoacoustic Streaming (PIPS) (Peters *et al.*, 2011). This system proposes the use of an Er:YAG laser with a specifically designed radial and stripped tip and recommends that the

tip be positioned in the pulp chamber and that the laser is activated at subablative parameters (average power 0.3 W, 20 mJ at 15 Hz) (Olivi *et al.*, 2014).

1.4.2 Cavitation and its mechanism of action in the root canal

Cavitation is ‘‘the formation of an empty space (bubbles) in a liquid’’ (Matsumoto *et al.*, 2011). Laser induced cavitation has been known for many years using lasers in the erbium family (Levy *et al.*, 1996). Blanken & Verdaasdonk (2007) were the first authors to show high-speed imagery of this phenomenon occurring within the confines of a simulated root canal that was filled with water.

Erbium lasers, such as the erbium, chromium:yttrium-scandium-gallium-garnet (Er,Cr:YSGG) laser, emit energy in pulses of approximately 130 µs duration. At the start of the laser pulse, photon energy is absorbed in a 2-10 µm thick layer that is instantly superheated to become a highly pressurised vapour bubble. As the laser continues to emit energy, the expanding bubble creates an opening in front of the laser fibre thereby allowing the laser light to pass through the bubble and evaporate the water surface at the front of the bubble (Blanken *et al.*, 2009). This process continues like a “drill” until the laser pulse ends. This mechanism has been referred to as “the Moses affect in a microsecond region” (van Leeuwen *et al.*, 1991).

As the vapour bubble expands, a low pressure environment is created within the bubble compared to the surrounding liquid. After a laser pulse ends, the vapour bubble begins to cool and condense. To return to equilibrium, the bubble begins to implode near the laser fibre tip thereby causing the surrounding liquid to accelerate into the gap. This process continues until implosion of the bubble is complete and the bubble has vanished. This whole process of expansion and implosion occurs with each pulse of the laser in a free water environment. Laser induced cavitation and implosion is best visualised in a large volume of liquid (Figure 1). Confined between the root canal walls, the vapour cannot expand freely in a lateral direction, hence the fluid is pushed forward and backward in the canal (Matsumoto *et al.*, 2011) (Blanken & Verdaasdonk, 2007) (Blanken *et al.*, 2009).

After implosion of the vapour bubble, secondary cavitation bubbles are formed (Blanken *et al.*, 2009). This rebound phenomenon may continue multiple times. In a glass

cylinder simulating a root canal, Matsumoto *et al.* (2011) showed that cavitation followed by implosion occurred up to 10 times per laser pulse although the bubbles became progressively smaller with each cycle.

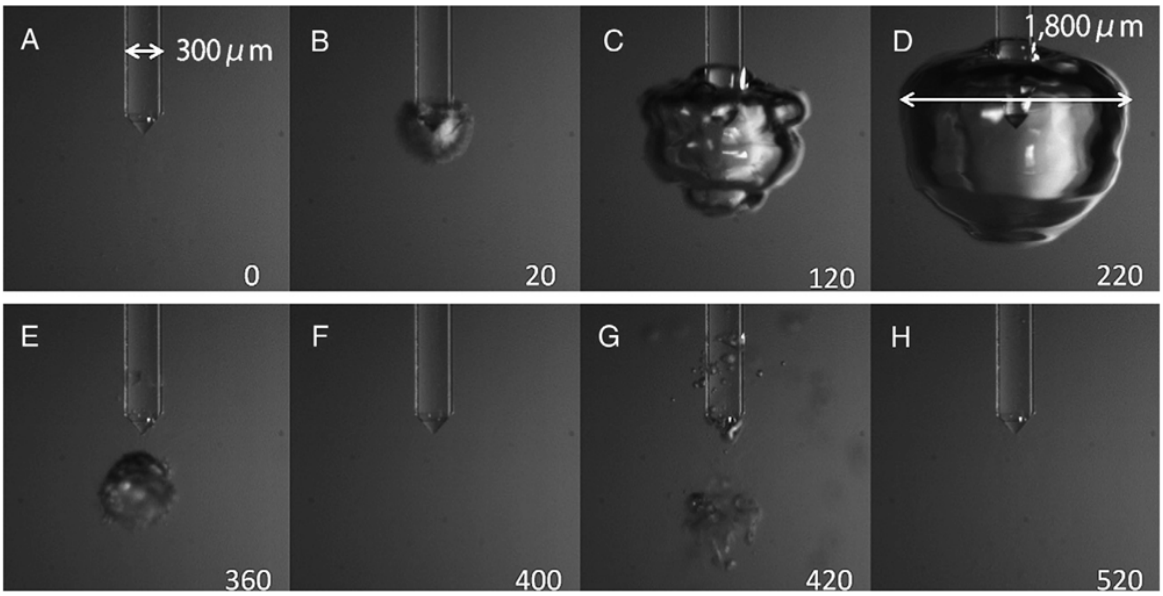

Figure 1. Images from Matsumoto *et al*. (2011). Laser induced cavitation and implosion. Numbers show the time interval (microseconds) from the beginning of the laser pulse: a) laser fibre immersed in water, b) initiation of vapour bubble, c) vapour bubble increasing in size, d) largest vapour bubble, e) implosion, (f) vapour bubble disappears, g) secondary cavitation effect, h) vapour bubble disappears.

When the vapour bubble begins to collapse, pressure waves are generated. Initially these pressure waves travel at supersonic speed and then later at sonic speed. Liquid jet streams are also created next to the imploding bubble surface. The pressure waves and the rapidly moving liquid cause high shear stresses along the canal wall (Matsumoto *et al.*, 2011). These forces may remove debris and the smear layer from the canal wall and aid the cleaning process (George & Walsh, 2008). Fluid turbulence is created both in front of and behind the laser tip when laser cavitation occurs within the root canal (Matsumoto *et al.*, 2011). By increasing the power output, the turbulence may be increased (George & Walsh, 2008).

1.4.3 Types of lasers

Lasers create monochromatic light, i.e. they produce light in a single wavelength (Peters *et al.*, 2011). The wavelength produced by a laser is determined by the active medium such as erbium (Parker, 2007). Erbium lasers, such as the Er,Cr:YSSG and the erbium:yttrium-

aluminium-garnet laser (Er:YAG), are solid-state lasers which are classified as middle infrared lasers and emit wavelengths (Er:YSGG 2780 nm, Er:YAG 2940 nm) that corresponds closely to the absorption maximum of water (George & Walsh, 2008; Olivi *et al.*, 2014). This means that the majority of the energy emitted by an erbium laser is absorbed and transferred into heat. This is also true for irrigants (e.g. NaOCl) with absorption coefficients similar to water (Parker, 2007). As previously mentioned, for cavitation to occur, the laser energy must be absorbed efficiency and cause a thin layer of fluid near the laser tip to be instantly vaporised (Blanken & Verdaasdonk, 2007). For this reason, Er,Cr:YSSG and Er:YAG lasers are commonly used for LAI.

Certain diode lasers (semiconductor lasers) have wavelengths of 940 and 980 nm (near infrared) that are close to the harmonics of water (Gutknecht *et al.*, 2004). Although their wavelengths are less strongly absorbed by water than erbium lasers, they have been shown to induce cavitation (Hmud *et al.*, 2010) and agitation in aqueous fluids that increases the effectiveness of certain endodontic irrigants such as EDTA (Lagemann *et al.*, 2014). Hmud *et al.* (2010) showed that in distilled water, cavitation could be achieved within 5 seconds when the panel setting was set to a power level of 2.5 W/25 Hz (actual power at the fibre tip was 1.35 W) for the laser with a 980 nm wavelength whereas a power setting of 4 W/10 Hz (actual power at the fibre tip was 1.68 W) was needed by the diode laser with a 940 nm wavelength. Cavitation was also shown to occur in 3% hydrogen peroxide.

1.4.4 Laser power setting

The power output of a laser is measured in Watts (W) and is defined as joule per second (W=J/s). It can be used to describe the energy (measured in J or mJ) conversion rate with respect to time. The pulse repetition is measured in hertz (Hz), where 25 Hz equals 25 pulses per second. For example, an output power setting of 0.75 W at 25 Hz has pulse energy of 30 mJ (0.75/25 = 0.03 J = 30 mJ).

Erbium lasers have the ability to produce both high and low levels of energy. High energy levels are needed for ablation of hard tissue such as dentine (Raucci-Neto *et al.*, 2014) whereas cavitation can be achieved within simulated root canals using relatively low subablative powers (Blanken *et al.*, 2009). Blanken & Verdaasdonk (2007) showed cavitation could occur at 12.5 mJ at 20 Hz (0.25 W) using an Er,Cr:YSSG laser. Seet *et al.*

(2012) using the same laser parameters as Blanken & Verdaasdonk (2007) showed that bacterial reduction was improved when 4% NaOCl was laser activated compared to conventional syringe irrigation.

Higher power outputs are needed to produce cavitation when the wavelength is not strongly absorbed by the irrigant. George *et al.* (2014) recently showed that a diode laser, set at a power setting of 2 W, was capable of initiating cavitation within approximately 5 to 15 seconds and that the time needed to cause cavitation was dependent on the design of the fibre tip. Increasing the power output decreased the time needed to initiate cavitation. The time needed to cause cavitation using a diode laser is considerably longer than that required for erbium lasers which are in the range of 1 µs to 1 ms. In small diameter canals, a longer activation time is needed before cavitation begins because the vapour bubble is not able to expand as easily as in a large volume of liquid (Blanken & Verdaasdonk, 2007) .

1.4.5 Laser fibres

Various laser tips have been designed and investigated. Initially, plain/end firing fibres were investigated and have been shown to be effective in causing cavitation and fluid flow in a relatively linear pathway. Extrusion of irrigant into the periapical tissues is a concern. Within the confines of a typical root canal, end firing fibres may cause irrigant to flow at high velocities (~ 30 m/s) (Blanken & Verdaasdonk, 2007) and it has been shown that the risk of apical extrusion is increased as the laser fibre tip is moved towards the apex (George & Walsh, 2008). To counteract the risk of extrusion and to redirect the shock waves into the canal wall and isthmus regions, radial and conical end firing fibre tips have been developed (George *et al.*, 2008; DiVito *et al.*, 2012; George *et al.*, 2014). The diameter of the laser fibre may also vary (200, 320 and 400 µm) with smaller diameter fibre being more flexible and suitable for small diameter root canals. The diameter of the fibre may also influence the length of the initial cavitational event when similar energy settings are used (Blanken & Verdaasdonk, 2007).

1.4.6 Irrigants

Cavitation has been shown to occur in different solutions such as distilled water,

NaOCl, EDTA, hydrogen peroxide and others (George *et al.*, 2008). Although cavitation and implosion may appear visually different in different irrigants, (de Groot *et al.*, 2009) LAI does not dramatically change the fundamental properties and working mechanism of the irrigating solution. For example, LAI using distilled water may be slightly more effective at removing the smear layer than water delivered by a syringe, however the improvement is negligible when compared to conventional syringe irrigation using a chelator (George *et al.*, 2008).

1.4.7 Smear layer removal

Laser activated irrigation may assist in the removal of the smear layer. George *et al.* (2008) showed that laser activated irrigation significantly improved the action of EDTAC on removing the smear layer from the apical third of root canals compared to traditional syringe irrigation using a combination of NaOCl and EDTAC. They also found no significant difference in smear layer removal between two commonly used erbium (Er,Cr:YSSG and Er: YAG) lasers. Laser activation of 3% hydrogen peroxide did not improve smear layer removal compared to syringe irrigation with EDTAC.

Peeters & Suardita (2011) confirmed that laser activated EDTA improves smear layer removal compared to 60 seconds of ultrasonic activated irrigation. Like George *et al.* (2008) they activated the Er,Cr:YSSG laser at a high power setting of 1 W using an end firing fibre (600 µm diameter) however they positioned the tip in the pulp chamber and did not enter the canal. This showed that LAI effectively disperses EDTA to the working length however it is not known whether apical extrusion will occur using high power settings. DiVito *et al.* (2012) used an Er:YAG laser with a low power setting (20mJ 15 Hz) in comparison to (George *et al.*, 2008) and Peeters & Suardita (2011). They showed that smear layer removal was improved by laser activation of EDTA compared to syringe irrigation. They also showed that smear layer removal was improved when activation time was increased from 20 to 40 seconds.

Recently Lagemann *et al.* (2014) showed that smear layer removal was significantly enhanced when a near infrared diode laser was used to activate EDTA compared to syringe irrigation methods. Although not tested in their study, the risk of extrusion is far less likely to occur with near infrared diode lasers compared to erbium lasers. This is because near

infrared diode lasers move fluid at about 4-5 mm/s (George *et al.*, 2014) compared to ~30 m/s for erbium lasers (Blanken & Verdaasdonk, 2007).

Sodium hypochlorite is effective at removing pulpal debris and pre-dentine from root canal walls however it is relatively ineffective at removing inorganic components of the smear layer in comparison to EDTA (Baumgartner & Mader, 1987). It has been proposed that laser activation of NaOCl may improve its smear removing capabilities, thereby negating the need to use a chelating agent. Moon *et al.* (2012) obturated root canals with different irrigating protocols and assessed sealer penetration depth using confocal laser scanning microscopy. They found that laser energised 5.25% NaOCl was more effective than syringe irrigation using 5.25% NaOCl or 17% EDTA at improving sealer penetration into the dentine tubules. Although this is an indirect method of assessing smear layer removal and relies on the assumption that sealer will penetrate dentine tubules, it does indicate that laser activation of NaOCl does improve smear layer removal which may decrease the need for using a chelating solution before obturation.

Zhu *et al.* (2013b) confirmed that laser activation (using the PIPS protocol) of 3% NaOCl was as effective as conventional syringe irrigation using alternating rinses of 3% NaOCl and 17% EDTA. Although both groups successfully removed the smear layer in the coronal and middle third, neither groups effectively eliminated the smear layer in the apical third. Unfortunately they did not assess whether smear layer removal could be improved in the apical third by laser activating both NaOCl and EDTA.

All studies in this area have used teeth with relatively straight single canals. Further research is needed to see if laser energy can be utilised to improve smear layer removal in teeth that have curved narrow canals with complex anatomical features. New technology and treatment protocols will most likely be incorporated into clinical practice if they are not prohibitively expensive, safe to use and can provide substantial improvements in teeth with complicated root canals systems that are not cleanable using current treatment modalities.

1.4.8 Debris removal

Removal of infected debris from the root canal system is important because improved

clinical outcomes are expected when there is less bioburden remaining within the root canal system (Rôças *et al.*, 2013). De Moor *et al.* (2009) used a pre-established debris model (Lee *et al.*, 2004a) to compare debris removal from the apical third. They activated 2.5% NaOCl for 20 seconds by either a laser or ultrasonic device. Laser irradiation was performed using an Er,Cr:YSSG laser (power setting of 1.5 W) with the tip of the end firing fibre being positioned 5 mm away from the apex. Laser activated irrigation was shown to be far more effective at removing the artificially packed debris compared to ultrasonic activation. These findings were confirmed by de Groot *et al.* (2009) using both an Er,Cr:YSSG and Er:YAG lasers. Interestingly, in a later study, De Moor *et al.* (2010) showed that UAI was just as effective as LAI when the number of UAI cycles (20 seconds of activation followed by 2 mL irrigation with 2.5% NaOCl) was tripled. This indicates that both treatment modalities may improve debris removal from areas located outside the main lumen of the canal. To achieve comparable results, ultrasonic activation requires both an increase in irrigant volume and activation time.

Deleu *et al.* (2013) in a similar study to De Moor *et al.* (2009), investigated debris removal from the apical third using 2.5% NaOCl and a variety of different irrigating methods. They found that all irrigating activation methods removed more debris than syringe irrigation. The Er:YAG laser (power setting of 1.2 W), fitted with an end firing fibre, positioned 5 mm away from the apex, was more effective than activation with a Er:YAG laser and PIPS tip (positioned in the pulp chamber). No significant difference was found between the Er:YAG laser and the UAI treatment groups.

Debris removal from lower molar mesial roots was assessed recently by Lloyd *et al.* (2014) using x-ray microfocus computed tomographic imaging. After chemomechanically preparing the roots to a size 30/0.06 they performed laser activated irrigation using the PIPS protocol. The PIPS protocol removed 2.6 times more debris than syringe irrigation using a combination of EDTA and NaOCl. This study by Lloyd *et al.* (2014) is the only study that has assessed the efficacy of removing debris from canals using LAI with known complex anatomical features. More studies are needed that compare this new technology with other adjunctive irrigating methods in teeth with complex anatomical features.

1.4.9 The efficacy of laser activated irrigation

Peters *et al.* (2011) was the first study to assess the antimicrobial effect that may occur with the activation of NaOCl using either laser or ultrasonic energy in comparison to conventional irrigation using a syringe and needle. Extracted single rooted human teeth were standardized, sterilised and contaminated *in situ* to establish a mature three week old biofilm. All canals were initially irrigated for 30 seconds using 6% NaOCl using a syringe and needle positioned within close proximity of the working length. The roots were then either rested for a further 30 seconds or treated using either UAI or LAI (PIPS protocol; Er:YAG laser (50 mJ 10Hz) with radially 400 μm fibre tip being positioned in the coronal 5 mm of the canal). Microbial sampling was performed using traditional paper point sampling methods. They found that 50% (10/20) of samples were culture negative when the roots were treated using LAI. For the UAI and syringe irrigation groups, 2 and 20 samples respectively were culture negative. Statistical analysis showed that LAI was superior to UAI and syringe irrigation. When mean bacterial reduction was used as the measure to determine treatment efficacy, no statistical difference was found between the treatment protocols.

The findings reported by Peters *et al.* (2011) have been used by other studies (Al Shahrani *et al.*, 2014; Neelakantan *et al.*, 2014; Olivi *et al.*, 2014) to advocate the superiority of LAI (PIPS) in comparison to both UAI and syringe irrigation however little attention has been given to how well the PIPS protocol reduced bacterial levels in the roots that were positive for bacteria. Peters et al. (2011) did not find a statistical difference between the median and mean CFU/mL values. This may indicate that even if a difference does exist between the groups the difference may be clinically insignificant.

The median and mean CFU/mL values for all treatment groups were all greater than 2 X 10^6. This indicates that the contact time between NaOCl and the intraradicular biofilm was too short. Increasing the irrigation and activation times would most likely have improved the efficacy in all groups. This is because the efficacy of NaOCl in eradicating bacteria in a biofilm is partially dependent on contact time, availability of active chlorine ions (Berber *et al.*, 2006) and mechanical agitation (Harrison *et al.*, 2010). Short treatment times may favour the use of LAI because of the increased reaction kinetics (Macedo *et al.*, 2010). This study by Peters *et al.* (2011) does show that LAI may be of clinical benefit and warrants further investigation.

Seet *et al.* (2012) established a mature *E. faecalis* biofilm in straight roots with single canals. They grew the biofilm in a flow cell for four weeks before treating the roots using either syringe irrigation, sonic agitation (EndoActivator, Dentsply) or LAI (Er,Cr:YSGG laser (0.25 W, 20 Hz), with an end firing fibre. Canals were irrigated with 5 mL of 4% NaOCl over a 10 second period before the irrigant was either rested (syringe irrigation), sonic or laser activated for 5 seconds. This process was repeated four times giving a total treatment time of 60 seconds. Qualitative assessment using SEM analysis showed that LAI resulted in the greatest bacterial reduction compared with the other two groups.

Pedullà *et al.* (2012) grew a 15-day-old *E. faecalis* biofilm in *ex vivo* roots. They treated the biofilm by syringe irrigation using 3 mL 5% NaOCl or LAI (PIPS protocol) over 30 seconds. Twenty five of 32 teeth were found to have negative cultures in the syringe irrigation group whereas 30 were found to have negative cultures in the LAI group. For reasons unstated, they only examined serial dilutions of 10^6 to 10^4 and recorded a positive culturing result if more than 30 colonies were found in the 10^4 dilution. This means that all samples with less than 3 X 10^6 CFU/mL were counted as negative samples. Their methodology is questionable, and brings uncertainty about their findings that no significant difference was found between treatment protocols.

Zhu *et al.* (2013b) grew a 4 week *E. faecalis* biofilm in single rooted teeth before treating the roots with either conventional syringe irrigation using 10 mL 3% NaOCl or LAI using 3 mL 3% NaOCl before laser activation for one minute. They used the PIPS protocol although they did not supply a continual flow of irrigant into the pulp chamber but added irrigant if it became low. No significant difference was found between the groups. SEM analysis revealed no significant difference between syringe irrigation with NaOCl or NaOCl + EDTA and the LAI (PIPS) treatment protocols.

Zhu *et al.* (2013b) position the 30-gauge NaviTip safety needle within 1 mm of the working length. They suggested that LAI may be advantageous in small canals however if the conventional needle is able to reach the apical third, then the protocols would have similar disinfecting abilities. Macedo *et al.* (2010) showed that chlorine ions are quickly consumed by reaction with organic substances (e.g. biofilm) when NaOCl is laser activated. Given that the NaOCl was not replenished during laser activation it is possible that the chlorine ions were quickly consumed thereby reducing exposure time and reducing

the antibacterial effect. If a continuous supply of irrigant had been applied during activation, this may have improved the efficacy and a significant difference may have been seen.

Neelakantan *et al.* (2014) investigated the efficacy of syringe irrigation, UAI and LAI in eradicating a four-week-old *E. faecalis* biofilm. Laser activation was performed using either an Er:YAG laser (PIPS protocol) or a Ezlase (Biolase) diode laser (940 nm wavelength, 50-60Hz, 7W continuous wave mode, 200 μm plain ended fibre). They used 3% NaOCl as the antibacterial solution alone or in sequence with various chelating agents (18 % etidronic acid or EDTA). Unlike studies before them which activated the solutions for a total time of 20 (Seet *et al.*, 2012) and 60 seconds (Zhu *et al.*, 2013b), they activated the solutions in 30 second cycles, after which the irrigants were refreshed. This process was repeated until a total activation time of 4 minutes was reached.

Neelakantan *et al.* (2014) took dentine shavings from the canal wall and found complete eradication of *E. faecalis* up unto a depth of 200 μm using either UAI or LAI and all combinations of irrigating solution. Laser energised irrigation was only shown to be statistically superior to UAI (at 400 μm depths) when a mixture of NaOCl and etidronic acid was used as the irrigant. No statistical difference was seen however between the treatment protocols when NaOCl-EDTA-NaOCl regimen was used. The continuous chelating effect of the etidronic acid may have improved the efficacy of this irrigating regimen. Neelakantan *et al.* (2014) also used confocal laser scanning microscopy to assess the efficacy of the treatment protocols. Passive ultrasonic irrigation was found to be inferior to LAI when NaOCl + etidronic acid or NaOCl-EDTA-NaOCl irrigating regimes were used.

This study by Neelakantan *et al.* (2014) shows that syringe irrigation is effective in roots with straight canals if a chelating agent is used in combination with NaOCl. To achieve high levels of bacteria reduction, the NaOCl must be in contact for extended periods of time (e.g. 4 minutes). Further bacteria reduction can be achieved within the dentine tubules to a depth of 400 μm if the irrigant is activated by ultrasonic or laser energy however this is also dependant on the combination of solutions used. The small differences between the groups may not be clinically relevant.

Al Shahrani *et al.* (2014) mechanically prepared single canal teeth to a size 25/0.08 growing a mature *E. faecalis* biofilm. They found that LAI (PIPS) combined with 6% NaOCl was more effective at removing mature *E. faecalis* biofilm than syringe irrigation using a 30-gauge needle. Interestingly, needle position was not mentioned, despite their detailed methodology. This seems to be a convenient omission of information, especially given that one of the researchers and co-authors (De Vito) declared a financial interest in the PIPS technology. The risk of research bias is therefore high.

Another recent study by Olivi *et al.* (2014) (De vito was a co-author) found that PIPS combined with 6% NaOCl was more effective at removing four week old *E. faecalis* biofilm from the root canal than syringe irrigation using a 30-gauge needle. The needle was placed in the mid root region in this study whereas it could easily have been placed to within 1 to 2 mm of the apex given that the canal had been prepared to an apical size of 25 and a 0.06 taper. If the needle had been placed within the apical 1 to 2 mm of the canal, it may have been possible to overcome the limitations of conventional irrigation (Tay *et al.*, 2010) and thereby improve the efficacy of the syringe irrigation protocol (Sedgley *et al.*, 2005b).

In a similar study to Seet *et al.* (2012), Christo (2012) established a mature *E. faecalis* biofilm in *ex vivo* single canal roots. They treated the roots with either LAI (Er,Cr:YSGG laser) or syringe irrigation using different concentrations of NaOCl and longer activation time. Seet *et al.* (2012) used a power setting of 0.25 W (12.5 mJ, 20Hz) whereas Christo (2012) used a power setting of 0.5 W (25 mJ, 20 Hz). Each canal was irrigated with 1.25 mL of NaOCl before being laser activated for 15 seconds. The cycle was repeated four times thereby giving a total activation time of 60 seconds. The results showed that LAI using 1% NaOCl was significantly more effective at reducing bacteria levels compared to syringe irrigation using 1% NaOCl. No difference was seen between LAI using 4% NaOCl and syringe irrigation using 4% NaOCl. They also showed that LAI using 1% NaOCl was just as effective as syringe irrigation using 4% NaOCl. This result shows that lower concentrations of NaOCl may be activated to give similar antibacterial effects as higher concentrations of NaOCl. This may have important clinical implications given that lower concentrations of NaOCl are less toxic (Zhu *et al.*, 2013a).

A recent study by Ordinola-Zapata *et al.* (2014) used SEM to investigate the efficacy of

different irrigating protocols. They treated 10 day old *E. faecalis* biofilm using LAI (PIPS protocol) and 6% NaOCl with a total activation time of 60 seconds (3 x 20 second activation). They found that UAI, sonic agitation or syringe irrigation were inferior to LAI which confirmed the findings of Seet *et al.* (2012). Juric *et al.* (2014) showed that LAI significantly reduced bacteria number (CFU/mL) from infected canals compared to UAI and other irrigating methods. They however used an Er,Cr:YSGG laser, (1.5 W, 20 Hz) with an end firing tip which was positioned 5 mm away from the apex of the tooth. Although these results seem to show that LAI may improve the removal of bacteria from the root canal system, it is unknown whether these parameters are safe to use in a clinical setting.

1.5 Rationale of this study

Current chemomechanical procedures substantially reduce bacterial load however they are unable to predictably eliminate bacteria from the entire root canal system. Different supplementary irrigating methods have been advocated that use ultrasonic or laser energy to improve the cleaning and disinfection of the root canal system. Ultrasonic activated irrigation uses acoustic microstreaming and cavitation to improve irrigant dispersion and debris removal from the root canal system. Laser activated irrigation is thought to improve cleaning by laser-induced cavitation that sends powerful shockwaves through the irrigant, while also causing high speed streaming (Matsumoto *et al.*, 2011) and an increase in an irrigants chemical efficiency (Macedo *et al.*, 2010).

Ultrasonic devices are relatively inexpensive, have widespread acceptance and may be easily incorporated into the irrigating protocol to improve the efficacy of the chemomechanical procedures. Laser activated irrigation has shown promising preliminary results however erbium lasers are currently expensive, used by few clinicians and safety concerns exist.

To date, no study has investigated the efficacy of LAI using teeth with complex root canal systems. Perhaps laser activated irrigation when used as a supplementary approach to traditional chemomechanical procedure will be more effective than UAI at improving the eradication of bacteria from the main root canal, dentine tubules and complex anatomic features such as lateral canals, isthmi, fins and webs. LAI may be the answer to improving

the removal of mixed species biofilm from teeth with complex root canal systems.

1.6 Aim

The aim of this study was to investigate the efficacy of laser activated irrigation and ultrasonic activated irrigation on eradicating a mature mixed species biofilm grown in the mesial roots of human mandibular molars.

The null hypothesis was that no difference would be found between LAI and UAI on eradicating a mixed species biofilm from the mesial roots of human mandibular molars.

2 Materials and Methods

2.1 Tooth collection and storage

This study was approved by ethics committees from The University of Adelaide (H-2012-076), Royal Adelaide Hospital (120511), and the South Australian Dental Service. Lower first and second molar teeth that were to be discarded after extraction were collected by the Oral Surgery Department at the Adelaide Dental Hospital and from private practices of participating oral and maxillofacial surgeons. After extraction, the teeth were washed in tap water to remove excess blood and stored until use in 0.02% (v/v) thymol in 0.9% saline (w/v). Before use, teeth were examined to ensure that the mesial roots were undamaged and had fully formed apices and root lengths greater than 11 mm. The teeth were then immersed in 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) for 10 minutes to remove the periodontal ligament (PDL) and excess tissue from the root surface.

2.2 Root preparation

The mesial roots of 96 teeth were removed from the crown and distal root(s) with a diamond coated disc (3M, Minnesota, USA) to obtain a standard root length of 11 mm. The canals were negotiated to achieve patency with sizes 6, 8 and 10 stainless steel K-files (SybronEndo, California, USA) until a glide path was established with a size 15 K-file. Mesial roots with more than two canals were discarded. Each canal was prepared in a crown down manner with SybronEndo Twisted Files (TF) to achieve a canal size that matched a final rotary TF instrument size of 25/04 (apical size 0.29 mm) 1 mm beyond the apical foramen. This size was selected to facilitate the flow of nutrient media through the canals and to support the growth of a mature biofilm. Approximately 3 mL of 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) was used to irrigate each canal during the preparation stage. After canal preparation was achieved, the canals were irrigated with 1 mL of 17% (w/v) EDTA for one minute to remove the smear layer ((Baumgartner & Mader, 1987)) and deactivate residual NaOCl ((Clarkson *et al.,* 2011)). The canals were then irrigated with 1 mL of 0.9% saline. All irrigation was performed using a 3 mL syringe and a 30-gauge Endo-Eze tip (Ultradent, Utah, USA) needle.

The external surfaces of the roots were dried with sterile gauze while the root canals

were dried with paper points. A size 20 paper point was placed firmly each in the canal so that approximately 5 mm of paper point was seen protruding through the apical foramen. This helped prevent occluding the apical foramen with nail varnish. A size 40 K-file was gently locked into the canal to facilitate handling of the tooth. A coat of nail varnish (Deeply Chili 741, Revlon, New York City, USA) was applied to the entire external tooth surface and allowed to dry before a second coat of different coloured nail varnish was applied. After drying, the tooth was held with sterile gauze while the size 40 K-file and paper points were removed. The canals were liberally flushed with sterile 0.9% saline and a size 15 K-file was used to ensure that the canals and apical foramen had not become occluded during the varnishing process.

2.3 Determination of root canal configuration

Canal configuration varies between molar mesial roots and might indicate the size of the isthmus region. The isthmus region is a problematic area to disinfect with traditional chemomechanical measures. In the clinical setting, basic canal configuration can be determined by simultateous placement of a file in each canal. Binding of the files with one another can indicate if the canals join within a common region of the canal or at the foramen. No binding of the files might indicate two separate canals.

In this study, the mesial roots were pooled into two major canal configurations based on whether the two mesial roots had separate apical foramen (2:2) or shared a common apical foramen (2:1). This was determined by placing a size 15 Hedstrom file (SybronEndo, California, USA) in one canal until it was seen protruding through an apical foramen. A size 15 K-file was then inserted in the other canal until it was observed to pass through either the same apical foramen (2:1) or a separate apical foramen (2:2) to the Hedstrom file. The canal configuration was noted before adjusting each file to a level where the tips of the files were equal with the external surface of the apical foramen.

2.4 Radiographic determination of root canal angle

The tooth root containing the two files was mounted in a custom-made device. This device consisted of a plastic specimen jar lid and periphery wax (Heraeus-Kulzer Hanau,

Germany) and was placed over the tube end of an intraoral X-ray machine. Three teeth roots were positioned in a bucco-lingual direction, parallel with the X-ray beam. A size two periapical radiographic film (Kodak, New York, USA) was positioned behind the root and held in place with a layer of periphery wax. The radiograph was exposed (70kVp, 10 mA) with a Belmont intraoral X-ray system (Belmont, New Jersey, USA). The film was developed using an automatic developing machine (Veloprex, Florida, USA), mounted and stored in a plastic radiographic sleeve until after all flow cell experiments were completed.

A monochrome photo was taken of each film using a Canon D1100 digital camera and 100 mm macro lens (Canon Australia, North Ryde, NSW). Using Microsoft Office Picture Manager software, the images were cropped (3 x 4 aspect ratio) and resized (1024 x 731 pixels) to make each root appear approximately the same length. The "Schneider angle" (Schneider, 1971) representing the degree of root curvature was determined for each canal using the software program "Image J" (1.47v) (Schneider *et al.*, 2012) by following the Hedstrom file and then the K-file along its course through the canal. The long axis of the canal was determined by drawing a straight line that followed the file in the coronal portion of the canal. A second line was drawn from the tip of the file - located at the apical foramen – to a point where the file began to curve away from the first straight line. The obtuse angle formed by the two lines was determined by the software program. The "Schneider angle" of the canal is the acute angle formed by these two lines. The larger of the two acute angles was chosen to represent the tooth root canal angle. The tooth root was classified as being either mildly curved (≤15 degrees), moderately curved (16-29 degrees) or severely curved (≥30 degrees) (Figure 2). The classification system was arbitrarily specified to simplify the comparison between root canal angle and the quantity of bacteria remaining in the tooth root after treatment.

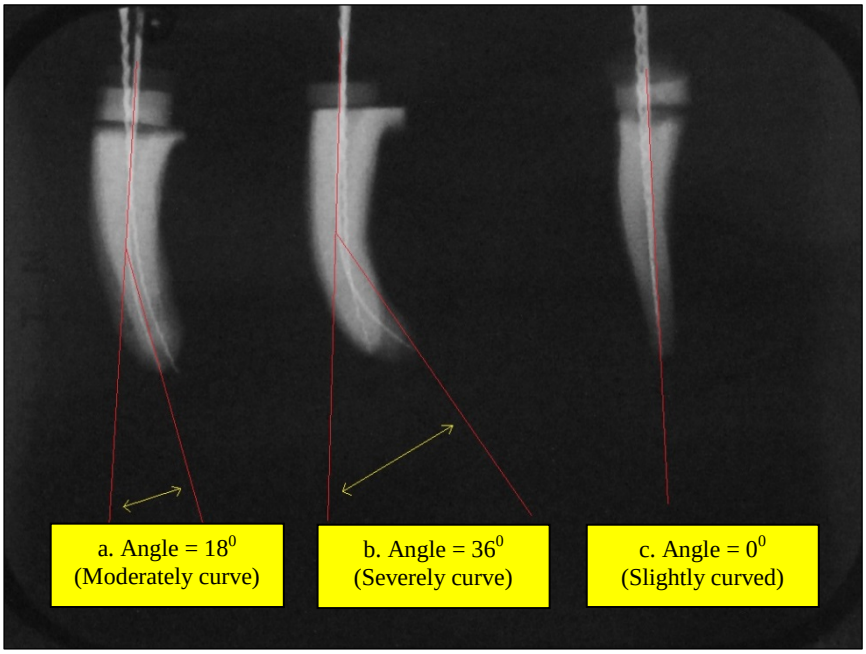

Figure 2. Radiographic image showing three mandibular molar mesial roots, each with a size 15 K-file and Hedstrom file in separate canals.
The Schneider angle is the acute angle formed by the two intersecting red lines. The roots are classified as either being slightly, moderately or severely curved.

2.5 The flow cell

This study used a continuous flow cell to establish a mature biofilm within decoronated teeth roots. The flow cell was designed by The University of Adelaide Endodontic research group and has previously been used for two independent research projects (Christo, 2012; Seet *et al.*, 2012).

The flow cell walls and base were fabricated from Delrin poly-acetyl resin (Dupont, Wilmington, DE, USA) measuring 200 mm (W) x 200 mm (L) x 60 mm (H). The top of the flow cell was made from clear Perspex to allow the internal compartments to be viewed. The flow cell had two sealed compartment that were separated by another clear Perspex plate. Twenty-four evenly spaced holes (10 mm in diameter) were placed in the Perspex to facilitate the mounting of tooth roots. Each Perspex hole was customised if the mesiodistal diameter of the root was greater than 10 mm. The Perspex plates were separated from the Delrin by two new neoprene gaskets and secured to the body of the flow cell with stainless steel screws (Figure 3). This provided a hermetic seal between the

external and internal flow cell environments while also establishing a hermetic seal between the upper and lower compartments.

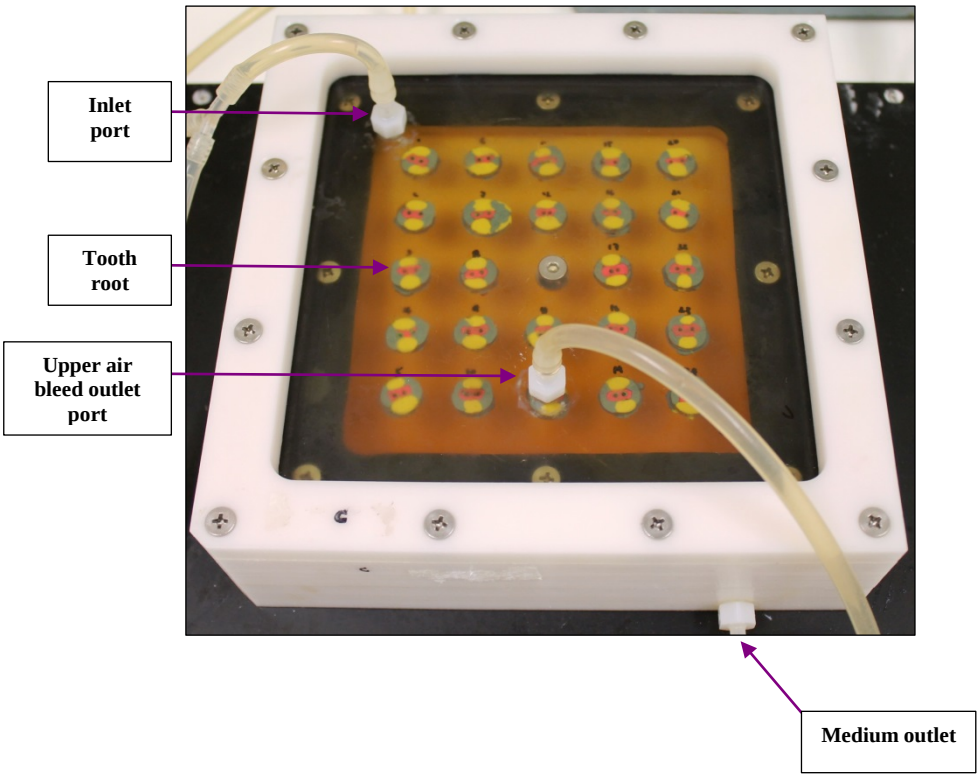

Figure 3. Sterilised flow cell containing 24 tooth roots, sealed in place with medium and light body impression material.
The teeth were immersed in sterile heart infusion broth before inoculation with bacteria. The top Perspex viewing plate contained an inlet port (top left) for nutrient media to flow into the flow cell and a second port (lower middle) for venting trapped air/ gases.

The top Perspex viewing window had two ports. The first port allowed growth medium to be pumped into the upper chamber of the flow cell while a second port allowed air to bleed from the flow cell during initial filling. The second port was also used to periodically remove gas produced by the biofilms during the experiment and also air that accumulated in the medium line when changing over the growth medium. When not in use this port remained closed via an attached sterile syringe. An outlet port in the lower chamber facilitated the removal of waste medium. This apparatus allowed a continuous flow of growth medium to be pumped into the top chamber of the flow cell, through the root canal and into the bottom chamber before exiting the flow cell via the outlet port.

2.6 Allocation and installation of tooth roots to the flow cells

Ninety-six tooth roots were divided into two groups based on foramen type. There were 44 tooth roots with separate foramen and 52 tooth roots with common foramen (Appendices 6.1). The roots were divided so that each of the four flow cells contained 24 tooth roots consisting of 11 tooth roots with separate foramen and 13 tooth roots with a common foramen. The tooth roots were then assigned a position in the flow cell without preference being given to the foramen type (Appendices 6.2).

Tooth roots were positioned in the Perspex plate with the coronal orifice facing the top chamber of the flow cell. Light and medium body polyvinyl siloxane impression material (3M ESPE, Seefeld, Germany) was then syringed around each tooth root to hold it in place (Figure 3). The top left corner of the flow cell next to the media inlet was designated as position one, whereas position 24 was located in the lower right corner next to the media outlet.

2.7 Sterilisation of the flow cell

After the tooth roots were mounted in the flow cells the flow cell was assembled and all inlets were covered with autoclave tape (Henry Schein, New York, USA). The flow cells were then individually wrapped in Kimguard sterilisation wrap (Kimberly-Clark, Texas, USA) before being sterilised by gamma radiation at 3-5 kGy (Steritech Pty Ltd, Dandenong, Victoria, Australia). This method has been shown to be an effective means of sterilising teeth (White *et al.*, 1994) and tooth roots in a flow cell (Seet *et al.*, 2012).

2.8 Experimental apparatus

The experimental apparatus consisted of a 2 litre Schott bottle (Schott, NSW, Australia) containing sterile heart infusion broth (HIB) (Oxoid, Victoria, Australia), sterile silicon tubing, peristaltic pump (Gilson, Middleton WI. USA), inoculation port, sterile flow cell containing tooth roots, slide dryer (Ratek, VIC, Australia) and waste vessel (2 litre Schott bottle). Figure 4 shows the assembled apparatus. Sterile Heart infusion broth was pumped to create a continuous flow of media from the nutrient vessel to the flow cell. Two sterile pyrex glass “breakers” were inserted in the silicon tubing between the peristaltic pump and the flow cell to prevent “back-growth” of bacteria in the nutrient feed-line. The flow cell

was placed on a slide dryer with an attached electronic thermometer to keep the flow cell and its contents at 37°C throughout the experiment and the inoculation port was sealed with a sterile 3 mL syringe.

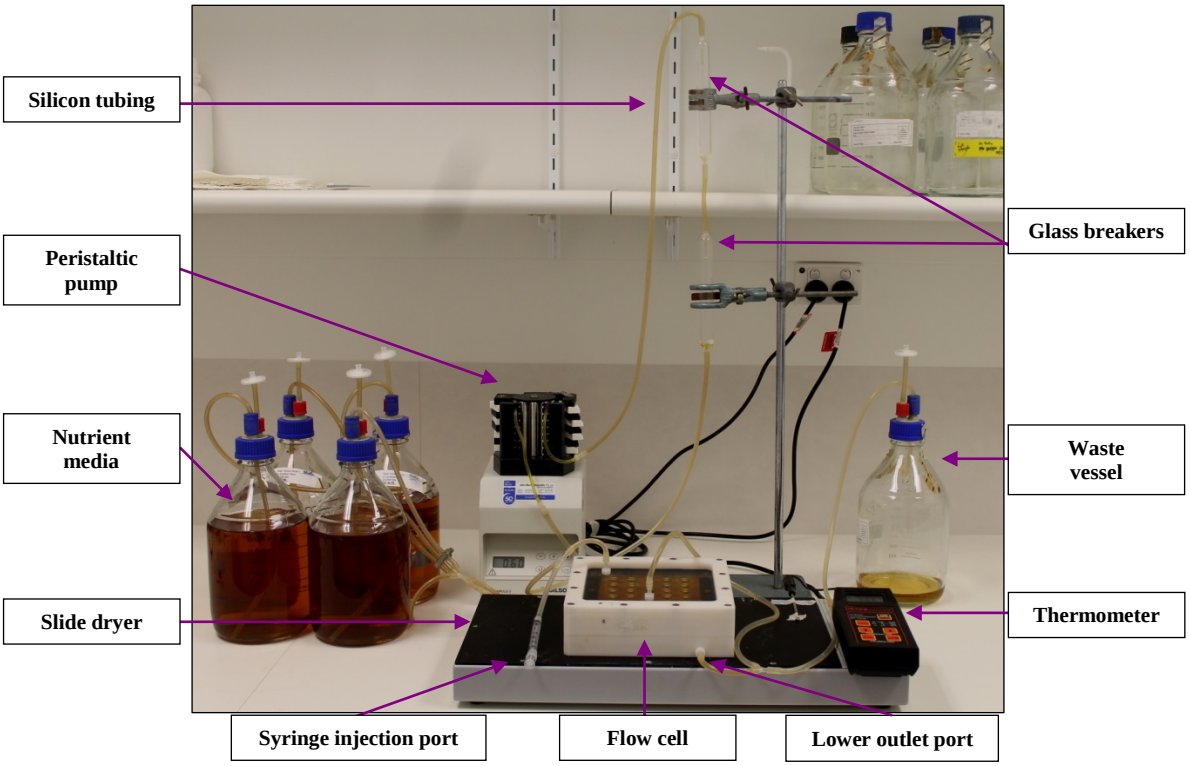

Figure 4. Experimental apparatus.

Heart infusion broth was prepared as per the manufacturing instruction. To support the growth of *F. nucleatum*, the HIB was supplemented with 40 mmol/L glutamic acid, 10 mmol/L lysine and 10 mmol/L histidine (Yap *et al.*, 2014). The nutrient media, silicon tubing, glass breakers and waste vessel were all connected. The free ends of the silicon tubing were covered with a sterilisation pouch and sealed with autoclave indicator tape. The equipment was sterilised by autoclaving at 15 psi at 121°C for 15 minutes before being aseptically connected to the flow cell. The flow cell was then filled with media and all air removed using the top chamber outlet port. The inlet and outlet lines were clamped and the flow cell left for 72 hours at 37°C to confirm sterility (the absence of turbidity).

2.9 Bacterial strains

Three bacterial species were chosen: *Enterococcus faecalis* (V583 ATCC 700802), *Streptococcus sanguinis* (P4A7) and *Fusobacterium nucleatum* (ATCC 25586). Yap *et al.*

(2014), a member of our research group, recently showed that these three species can be successfully grown as a mixed species biofilm in a flow cell. Stock cultures were stored frozen in 40% v/v glycerol at -80°C.

Before use, *E. faecalis* and *S. sanguinis* were cultured and maintained on HIB agar (Oxoid, Victoria, Australia) at 37°C. Species purity of *E. faecalis* was periodically checked by plating on bile aesculin agar (Oxoid, Victoria, Australia) and Gram staining. *F. nucleatum* was anaerobically cultured and maintained on anaerobic blood agar (Oxoid, Victoria, Australia) at 37°C in an anaerobic jar containing an atmosphere of 5% CO_2, 5% H_2 and 90% N_2.

2.10 Inoculation of the flow cell and biofilm growth

F. nucleatum was grown in 40 mL sterile HIB at 37°C for 48 hours under anaerobic conditions while *E. faecalis* and *S. sanguinis* were each grown independently in 40 mL of sterile HIB at 37° for 24 hours. Before inoculating the flow cell, the purity of each broth was checked by Gram staining (Figure 5).

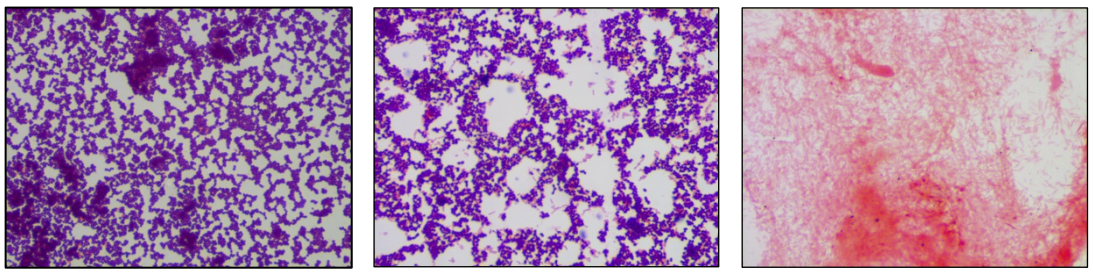

Figure 5. Digital photographs of three Gram stained slides to check the purity of broth cultures before inoculation of the flow cell.
a) *E. faecalis V583* (Gram-positive cocci), b) *S. sanguinis* P4A7 (Gram-positive cocci), and c) *F. nucleatum* 25586 (Gram-negative fusiform).

The flow cell was inoculated with 20 mL of *F. nucleatum* broth using a sterile syringe that was aseptically connected to the inoculation port. The flow cell was then allowed to grow as a batch culture for 48 hours before equal volumes (20 mL) of *E. faecalis* and *S. sanguinis* were added to the flow cell in the same manner. The flow cell was left for 24 hours before the peristaltic pump was activated. HIB was pumped at a continuous flow rate of 10 mL/hr (dilution rate of 0.017 hr^{-1}). The flow cell was checked on a regular basis to

ensure that the flow cell always had a continuous supply of sterile media. The flow cell was run continuously for 28 days to establish a mature mixed species biofilm within the root canal system. SEM analysis has shown that four weeks is an appropriate time frame for *E. faecalis* to form either a single (Plutzer, 2009; Christo, 2012; Seet *et al.*, 2012) or mixed species (Yap *et al.*, 2014) mature biofilm when grown on human dentine in a flow cell model. Figure 6 shows the flow cell upper chamber contents four weeks after inoculation with bacteria.

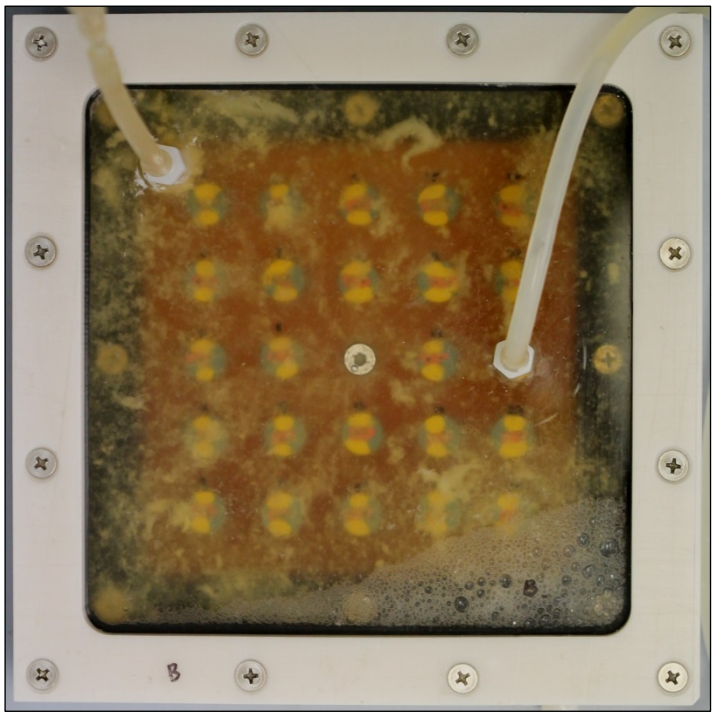

Figure 6. Flow cell upper chamber as seen through the Perspex viewing plate four weeks after inoculation with bacteria.
Thick biofilm deposits were dislodged from the Perspex plates when moving the flow cell from side to side to facilitate the regular removal of gases through to the upper outlet port (middle right).

2.11 Disassembly of the flow cell and preparation for endodontic treatment of tooth roots

After four weeks, the flow cell was drained and disassembled using aseptic techniques. The Perspex plate holding the roots was gently washed with 2 L of sterile 0.9% saline to remove any loosely attached biofilm and planktonic bacteria. The Perspex plate was then placed on sterile cloth. The tooth roots were removed with sterile gauze and placed in individually marked 7 mL sterile polypropylene tubes (Sarstedt, Adelaide, Australia)

containing 6 mL sterile HIB and stored at 37°C. The maximum storage time was 7 days. All roots were experimentally treated within 7 days following removal from the flow cell.

For logistical reasons, pre-treatment processing was done in small batches. Five to 10 tooth roots were removed from HIB storage and dried with sterile gauze. The coronal and apical orifices were sealed with Cavit (3M ESPE, Seefeld,Germany) and two coats of nail varnish (Deeply Chili 741, Revlon, New York City. After the nail varnish had dried, the tooth roots were placed in 7 mL tubes containing 3 mL of 4% NaOCl (Endosure, Dentalife, Ringwood, Australia). The roots remained immersed in 4% NaOCl for 75 minutes. The tubes were gently agitated every 20 minutes to facilitate the removal of bacteria from the root surface (Plutzer 2009). Sealing the orifices with Cavit and nail varnish prevented NaOCl from entering the root canal system.

The roots were then transferred to 7 mL tubes containing 3 mL 5% sodium thiosulphate to degrade residual NaOCl. After approximately 3 minutes, the roots were transferred to 7 mL tubes containing 3 mL of sterile 0.9% saline and stored at 37°C until treatment. The remaining teeth were processed the same way in small batches. Within one week of the flow cell being disassembled, treatment had been completed for all teeth.

2.12 Allocation of tooth roots to the treatment groups

The 96 tooth roots were allocated to one of 5 treatment groups with each group having similar proportions of roots with both foramen types. The number of teeth in each treatment group, foramen type and distribution among the flow cells is presented in the appendices (Appendices 6.2). A summary is presented in Table 1. One tooth root from each treatment group (1-5) was designated for SEM analysis.

Group	Treatment	Tooth root number
1	No treatment (No Tx)	8
2	Saline standard irrigation (Saline SI)	21
3	Ultrasonic activated irrigation (UAI)	21
4	Laser activated irrigation (LAI) at 0.5 W	23
5	Laser activated irrigation (LAI) at 0.75 W	23
	Total	96

Table 1. A summary of the number of teeth in each treatment group from the four flow cells (A, B, C, D).

2.13 Treatment protocols

A small (4 x 5 x 1cm) metal vice was modified to safely hold the tooth root during the treatment procedure. The vice was sterilized by autoclaving (15 psi at 121°C for 15 minutes). Heavy body impression material (3M ESPE, Seefeld, Germany) was placed over the vice plates to the prevent damaging the root. The vice was disinfected in 100% alcohol for a minimum of one hour before use and reuse.

Treatment was performed on one tooth root at a time. The tooth root was removed from saline storage with sterile tweezers and positioned in the vice so that the apical and coronal orifices were easily accessible. Approximately 5 mm of sticky wax was layered over the apical third of the tooth. This was done to mimic the *in vivo* root and surrounding periodontium where irrigating solution cannot flow freely through the apex. There is also potential for a vapour lock to occur with the apex sealed. The Cavit sealing the coronal orifice was removed with an ultrasonic scaler and tip (SybronEndo, California, USA). The ultrasonic tip was cleaned with a sponge and disinfected in 100% alcohol for at least one hour before reuse.

Group 1: No treatment (No Tx) (Control). (n = 7). The tooth roots were removed from saline storage and immediately crushed.

Group 2: Chemomechanical prepration using saline standard irrigation (Saline SI). (n = 20)**.** An ultrasonic scaler and tip was used to remove the Cavit sealing the coronal portion of the root canal. A size 15 K-file was used to remove Cavit debris, measure the working length of the canals and ensure the apical foramina were sealed. Canals were

sequentially prepared with SybronEndo TF rotary files sizes 25/06, 30/06 and 35/06 to working length. Between files, the canals were irrigated with 1 mL of sterile 0.9% saline and recapitulated with a size 15 K-file. All irrigation was performed using a 3 mL syringe and a 30-gauge Endo-Eze needle tip (Ultradent, Utah, USA). The needle was inserted into the canal to the point of binding and withdrawn approximately 3 mm. The canals were gently irrigated using parkinsonian movements.

Group 3: Chemomechanical preparation using 4% NaOCl and 15% EDTAC ultrasonic activated irrigation (UAI). (n = 20). As for group 2 but 4% NaOCl was used for irrigation during mechanical preparation of the tooth. The tooth was then subjected to an ultrasonic activated irrigation protocol using 4% NaOCl and 15% EDTAC as described below.

A size 20 SATELEC ®IrriSafe ™ file (Acteon, New South Wales, Australia) mounted in an ultrasonic device (SybronEndo, California, USA) was used on a medium power (7/15) setting. The IrriSafe file was placed into the canal to the point of binding and then withdrawn approximately 2 mm before being activated and gently moved to a position where the acoustics indicated minimal impediment from the canal walls.

Each canal was irrigated with 0.5 mL of 15% EDTAC for 15 seconds followed by ultrasonic activation for 15 seconds in each canal. This procedure was repeated once. It was then repeated another four times using 4% NaOCl as the irrigating solution. Overall, each canal was irrigated with 1 mL of 15% EDTAC and 2 mL of 4% NaOCl. To degrade the sodium hypochlorite, each canal was irrigated with 1.5 mL sterile 5% sodium thiosulphate. The tooth root was then placed in 3 mL sterile 5% sodium thiosulphate for three minutes.

Group 4: Chemomechanical preparation using 4% NaOCl and 15% EDTAC laser activated irrigation (LAI) at 0.5 W. (n = 22). As for group 3, however a Erbium, chromium: yttrium-scandium-gallium garnet (Er,Cr:YSGG) laser (Waterlase, Biolase Technology, Irvine, USA) and RFT 3 17 mm laser tip (Endolase, Biolase Technology, Irvine,) was used to activate the irrigating solution rather than an ultrasonic device and IrriSafe tip. To prevent the laser tip from descending more than 2 mm into the canal when activated, two rubber stoppers (removed from 15 K-files) were positioned 2 mm from the

end of the laser tip and secured in place with sticky wax. The air and water functions were turned off. The laser was set to a power setting of 0.5 W and a pulse repetition rate of 20 Hz.

Group 5: Chemomechanical preparation using 4% NaOCl and 15% EDTAC laser activated irrigation (LAI) at 0.75 W. (n = 22). As for group 4, however a laser output power setting of 0.75 W was used.

Scanning electron microscopy (SEM). (n = 5). One tooth root from each treatment group was designated for SEM analysis. After the appropriate treatment protocol, the tooth root was placed in SEM fixative (1.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose in PBS, pH 7.2) until further processing.

Immediately following treatment, all tooth roots except those roots preserved for SEM analysis, were crushed using the following protocol.

2.14 Crushed tooth sample preparation

The tooth was removed from the 5% sodium thiosulphate solution with sterile tweezers and placed in a sterile tooth crushing device (Seet 2012). This custom-made device consisted of a brass cylinder and stainless steel piston (Figure 7).

a) Brass cylinder and piston | b) Tooth root inside brass cylinder | c) Piston in cylinder

Figure 7. Tooth crushing device.
a) Brass cylinder and stainless steel piston b) Tooth root inside the brass cylinder ready for crushing c) The piston is inserted into the cylinder and hit forcefully with a hammer to crush the tooth.

The tooth was placed in the cylinder and the piston was hit with a hammer one to two times to break the tooth into a number of pieces. Half the sample was removed with sterile

tweezers and placed in sterile aluminium foil. One hundred microliters of 0.9% sterile saline was added to the cylinder to produce a thick slurry when crushed. The tooth fragments were crushed by forcefully hitting the piston 20 times. The tooth slurry was removed with a sterile spatula and placed in a sterile 7 mL tube containing approximately 20 sterile pink glass beads (approximately 1 mm in diameter). The remaining uncrushed sample was crushed in the same manner. Four millilitres of 0.9% sterile saline was added to the cylinder and agitated with a sterile spatula. A sterile 1000 µL pipette (with 5 mm of the tip removed) was used to transport the remaining cylinder contents to the tube containing the crushed tooth sample.

The piston and cylinder were washed with approximately 100 mL of sterile deionised water and dried with sterile gauze to remove any remaining debris. Nine sterile tooth crushing devices were available for use. The cylinders were sterilized by autoclaving at 15 psi at 121°C for 15 minutes and cooled to room temperature before being reused.

2.15 Sample separation and volume standardization

The tube containing the crushed tooth solution and glass beads was vigorously vortexed for 90 seconds to dislodge bacteria from the crushed tooth fragments into solution. The tube was centrifuged (20 X *g* for 60 seconds) to sediment the crushed tooth fragments. The supernatant was then transferred to another sterile 7 mL tube and centrifuged (8000 X *g* for 2.5 minutes). The supernatant was removed and placed in another sterile 7 mL tube and sample volumes were standardized by returning 1000 µL of the supernatant to the tube containing the bacterial pellet. The tube was then vortexed vigorously for 60 seconds to resuspend the bacterial pellet.

2.16 Cellular viability

Three hundred microliters was removed from the bacterial suspension and ten-fold serial dilutions were prepared. One hundred microliters of each dilution were plated in duplicate onto heart infusion agar plates (supplemented with 40 mM glutamic acid, 10 mM Lysine and 10 mM hisitidine) and incubated at 37°C for 48 hours.

After incubation the plates were photographed using a 5 megapixel digital camera (Apple Inc, CA, USA) at a standardised focal length (200 mm) and background light intensity. The digital images were transferred to the software program "OpenCFU" (Geissmann, 2013) (version 3.3) which counts and marks each colony that conforms to predetermined parameters (size and colour range) (Figure 8). The digital count was confirmed and adjusted after visually inspecting each image for any obvious counting errors. Colony-forming units (CFU) per mL were calculated for each sample.

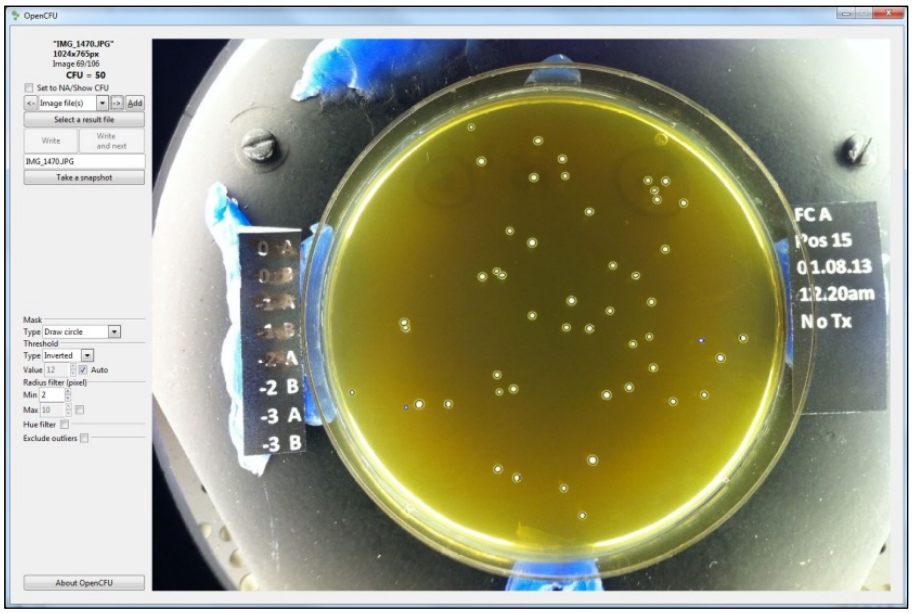

Figure 8. Screenshot image of the software program "OpenCFU".
The program was used to count *E. faecalis* and *S. sanguinis* colony-forming units. The software program counted 50 CFU (upper left corner of image) by marking each CFU with a blue ring. The image can be magnified to visually verify the calculated count.

2.17 Sample preparation for ultra-low temperature freezing

A backup cellular viability solution was prepared in case procedural errors or plate contamination occurred. This solution was prepared by placing 100 µL of sample bacterial suspension in a sterile 1.5 mL tube (Sarstedt, Ingle Farm, Australia) containing 100 µL of 80 % sterile glycerol. The tube was vortex and immediately placed at -80°C

The remaining 600 µL of the sample bacterial suspension was transferred to a labelled 1.5 mL tube and centrifuged (8000 X *g* for 2.5 minutes). The supernatant was carefully removed and 500 µL of RNA*later*® was added to the tube before being vortexed for 10 seconds. The tube was then placed at -80°C for DNA purification and qPCR analysis.

2.18 DNA extraction

DNA was extracted from bacteria using the ONE-4-ALL Genomic DNA Mini-Preps Kit (Bio Basics Canada Inc, Ontario, Canada). The manufacturer's instructions were followed however to decrease processing costs while increasing the concentration of the muralytic enzymes, the suggested protocol was scaled down to accommodate a 100 µl volume (Sedgley *et al.*, 2005c).

To facilitate bacterial cell wall degradation and DNA extraction from Gram-negative bacteria (*F. nucleatum*), an enzymatic lysis buffer was prepared as recommended by the manufacturer of the DNA extraction kit. Lysozyme (Sigma-Aldrich, New South Wales, Australia) purified from chicken egg white and Mutanolysin (Sigma) derived from *Streptomyces globisporus* (ATCC 2155), were also added to the solution to facilitate cell wall degradation of *E. faecalis* (Bibb & Straughn, 1962; Sedgley *et al.*, 2005c) and *S. sanguinis* (Yokogawa *et al.*, 1974) respectively. Each 100 µL of enzymatic lysis buffer contained: 20 mM Tris-HCL (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL of lysozyme and 15 µg of mutanolysin.

Frozen samples were thawed at room temperature and then centrifuged (8000 x *g* for 2.5 min) followed by careful removal of the RNA*later*®. The bacterial pellet was washed twice by adding 1 mL of PBS followed by vortexing, centrifugation (8000 X *g* for 2.5 min) and supernatant removal.

One hundred microliters of the prepared enzymatic lysis buffer was added to the bacteria pellet. The 1.5 mL tube was then vortexed (high setting) for 60 seconds and incubated at 37°C for 60 minutes. The incubation period was interrupted every 15 minutes to disperse the sample by votexing.

RNA was removed from the sample by adding 2 µL 100 mg/mL RNAse A (Qiagen, Hilden, Germany) followed by light vortexing and five minutes incubation at room temperature. Proteins and nucleases were removed from the sample followed by DNA extraction using the solutions and spin column provided by the manufacturer. The purified DNA was resuspended in 50 µL TE Buffer (Qiagen, Hilden, Germany) and stored at -20°C

until required.

2.19 Preparation of bacteria stock solutions used for qPCR verification assays

E. faecalis, S. sanguinis and *F. nucleatum* were grown separately as described previously in Section 2.9. For each bacterial species, 20 µl was used to inoculate 5 mL sterile HIB and incubated at 37°C until late exponential phase of growth. The optical density (600 nm) of the broth was periodically checked using visbile light spectroscopy (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA).

The final optical density reading for each broth was recorded (*E. faecalis* (OD=1.31), *S. sanguinis* (OD=1.37), *F. nucleatum* (OD=1.28) before 1 mL of each bacterial broth was transferred to sterile 1.5 mL tubes. The tubes were then centrifuged (8000 X *g* for 2.5 min) to pellet the bacteria before the supernatant was removed. To replicate the final processing steps of the crushed tooth samples, 500 µL of RNA*later*® was added to each tube before being vortexed and rested at room temperature for 5 minutes. The tubes were then placed in the ultra-cold (-80°C) freezer until required.

To determine if the qPCR assay could amplify bacteria from the flow cell, 1 mL of medium was removed from one flow cell after two weeks growth. Preparation of the bacteria for qPCR assays was the same as described for the other bacterial broth solutions. The stock solution was named “Flow cell C”.

2.20 Preparation of DNA stock solutions used for qPCR verification assays

Following DNA extraction and purification of the *E. faecalis*, *S. sanguinis*, *F. nucleatum* and Flow cell C broth solutions as previously described in Sections 2.18 and 2.19, the DNA concentration (A_{260}) and purity (A_{260}/A_{280}) were calculated using ultra-violet spectroscopy (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA). Each sample was then adjusted to a stock concentration of 5 ng/µl. All DNA stock solutions were stored at -20°C until required.

Equal volumes of *E. faecalis*, *S. sanguinis* and *F. nucleatum* 5 ng/µl stock solutions

were mixed together. This mixed species stock solution was used for validation and optimisation assays, standard curve construction, determination of the lower limit of detection and normalisation of all subsequent qPCR reactions.

2.21 Calculating 16S rRNA gene copy number in DNA stock solutions

Spectrophotometrically determined DNA concentrations were used to calculate the theoretical number of 16S rRNA gene copies in *E. faecalis, S. sanguinis* and *F. nucleatum* 5 ng/µL stock solutions. The number of genome base pairs (bp) for the *E. faecalis* V583 (3.36 Mb) and *S. sanguinis* SK36 (2.39 Mb) (*S. sanguinis* P4A7 has not been sequenced) and *F. nucleatum* 25586 (2.17 Mb) has been previously determined (http://www.cbs.dtu.dk/services/GenomeAtlas-3.0/). The genomic mass of a single cell may be calculated using the formula $m = n[1 \text{ mol}/6 \text{ X } 10^{23} \text{ (bp)}][660 \text{ (g)/mole}] = n[1.096 \text{ X } 10^{-21} \text{ (g/bp)}]$, where *m* is the genomic mass of a single cell and *n* the genome size (Rôças & Siqueira, 2012). The number of genome copies in 1 ng were then calculated (number of genome copies in 1 ng = (1 ng/*m*) where *m* is the genomic mass of a single cell.

The number of ribosomal RNA operons (*rrn)* differs between bacteria species. *E. faecalis* and *S. sanguinis* both have 4 copies whereas *F. nucleatum* has 5 copies of the 16S rRNA gene in each cell (http://www.cbs.dtu.dk/services/GenomeAtlas-3.0/). Thus, the calculated number of 16S rRNA gene copies in 1 µL of 5 ng/µL of each stock solution are presented in Table 2.

Bacteria	**Genome size in base pairs (n)**	**Genomic mass of a single cell (m)**	**Number of genome copies in 1 ng**	***rrn* operon number**	**Number of 16S rRNA gene copies in 1 µL of 5 ng/µL of stock solution**
E. faecalis V583	3.36 Mb	$3.68 \text{ X } 10^{-6}$ ng	$2.72 \text{ X } 10^{5}$	4	$5.45 \text{ X } 10^{6}$
S. sanguinis SK36	2.39 Mb	$2.62 \text{ X } 10^{-6}$ ng	$3.82 \text{ X } 10^{5}$	4	$7.65 \text{ X } 10^{6}$
F. nucleatum 25586	2.17 Mb	$2.38 \text{ X } 10^{-6}$ ng	$4.20 \text{ X } 10^{5}$	5	$1.05 \text{ X } 10^{7}$

Table 2. Theoretical determination of the number of 16S rRNA gene copies in 5 ng/µL of each stock solution.

2.22 Calculating 16S rRNA gene copy number in the mixed species stock solutions

The number of 16S rRNA gene copies in 1 µL of 5 ng/µL of mixed species stock solution was calculated by adding together the number of 16S rRNA gene copies in equal volumes of *E. faecalis*, *S. sanguinis* and *F. nucleatum* 5 ng/µL stock solutions before dividing by the total volume i.e. [(1 µL of *E. faecalis* (5.45×10^6) + 1 µL of *S. sanguinis* (7.65×10^6) + 1 µL of *F. nucleatum* (1.05×10^7) 16S rRNA gene copies / 3 µL) = 7.73×10^6 gene copies/µL].

Each PCR reaction contained 10 ng of template DNA. Therefore 10 ng of mixed species stock solution contained approximately 1.55×10^7 16S rRNA gene copies. This value was used for the construction of the mixed species standard curve.

2.23 Primer optimisation

Primers previously developed in our laboratory were used. The BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the validity of the primer set and ensure 100% nucleotide identity and coverage threshold. The primer set and their respective annealing temperatures were as follows: U16S forward, 5'-AGG ATT AGA TAC CCT GGT AGT CC- 3', 55°C (Tm); U16S reverse, 5'-CGT CAA TTC CTT TGA GTT TC- 3', 48°C (Tm). The approximate sizes of the expected PCR amplicons were 140 bp. The primers were sourced from Geneworks (Thebarton, SA, Australia).

To determine the validity of the primers, a qPCR assay was run using the mixed species stock solution and the individual species stock DNA solutions. The Ct value for each stock solution was compared against the respective theoretical 16S rRNA gene copy number (Table 2) to determine if the order of amplification was consistent with the theoretical values.

To determine the optimal primer concentrations, qPCR assays were carried out using the mixed species stock solution and varying primer concentrations. The forward (f) and reverse (r) final primer concentrations ratios were: 500 nM (f):500 nM (r); 500 nM (f):250 nM (r); 250 nM (f):500 nM (r) and 250 nM (f):250 nM (r).

Melting curve analysis was performed for all qPCR primer validation, primer

optimisation, standard curve and treatment group assays. Fluorescence acquisition occurred after each 1°C incremental temperature rise and a 5 second waiting period over a temperature range of 72 - 95°C. The melting curves were analysed to verify the reaction specificity and to minimise the chance of primer-dimer artefacts affecting the results.

2.24 Quantitative PCR conditions

Detection of bacterial DNA was performed with the Rotor-gene Q qPCR instrument (Qiagen). All qPCR reactions were performed in triplicate including a no template control. Each reaction was performed in a total volume of 20 µL which contained 1X Roto-Gene™ SYBR®Green PCR Master Mix (Qiagen), 250 pmol of forward and reverse primer and 10 ng of template DNA. Cycling conditions included 95°C for 5 min and 45 repeats of the following steps: 95°C for 5 seconds, annealing for 15 seconds at 60°C and extension and fluorescence acquisition for 20 seconds at 72°C.

2.25 Construction of the mixed species standard curve

The mixed species stock solution containing 1.55 X 10^7 16S rRNA gene copies, as described in Section 2.22, was ten-fold serially diluted. Quantitative PCR reactions were performed using triplicate samples. The standard curve was constructed using the Roto-Gene Q – pure Detection (version 2.1.0 (Build 9) analysis software. The threshold level was initially determined by the software's automatic threshold finder and then adjusted to slightly improve amplification efficiency (*E*) while marginally decreasing the correlation coefficient (R^2). The slope of the regression line and y-intercept value was also determined by the software program.

The lower limit of detection was determined as being represented by the last serial dilution that showed normal amplification curve profiles and congruency between the triplicate Ct values. The serial dilution representing the lower limit of detection was also checked against the no template control amplification curve profiles and Ct values to ensure there was no primer-dimer formation within a ten-fold range. Melting curve profile analysis was used to confirm the validity of each triplicate sample included in the standard curve.

2.26 Standardisation of qPCR runs and samples

The 10^{-3} standard curve serial dilution containing 1.55 X 10^4 16S rRNA gene copies was included in triplicate in all qPCR runs as a control. The threshold level for each qPCR run was set to the same level as determined by the standard curve analysis. Normalisation of each qPCR run against the standard curve was achieved by forming a standardisation run ratio calculated as: mean (10^{-3} standard) Ct value of qPCR run(*x*)/mean (10^{-3} standard) Ct value of the standard curve. This run ratio was applied to all treatment group sample Ct values in the run to give a standardised sample Ct value.

2.27 Determining 16S rRNA gene copy and bacteria cell number in treatment group samples

The absolute quantification method was used to determine the number of 16S rRNA gene copies for each treatment group sample by comparing the standardised Ct values with the slope of the standard curve. The 16S rRNA gene copy number mean (triplicate samples) was used to calculate the bacteria cell number in each treatment group sample.

It is impossible to determine the precise number of bacteria cells in a mixed species sample from 16S rRNA gene copy number when variations exist between the species quantity and operon number. For calculation purposes, the quantity of each bacterial species in a sample was considered equal, therefore the average number of gene copies was used, i.e. ([*E. faecalis* (4 gene copies) + *S. sanguinis* (4 gene copies) + *F. nucleatum* (5 gene copies)] = [13/3] = 4.3 gene copies/bacteria cell). The mean number of bacteria cells for each 10 ng of 2 µL qPCR reaction was calculated. The number of bacteria cells in 1 mL (cells/mL) of standardised crush tooth solution was then determined using the formula: number of bacteria in 1 mL (cells/mL) = [(number of bacteria cells in 2 µL) X (total volume of DNA elution) X ((total volume of crushed tooth solution)/(portion of crushed tooth solution used for DNA extraction))] = [(number of bacteria cells in 2 µL) X (25 µL) X (1000 µL/600 µL)].

2.28 SEM analysis

The five samples assigned for SEM analysis were stored in SEM fixative (1.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose in PBS, pH 7.2) for 4-8 weeks at 4°C. The tooth roots were longitudinally sectioned by placing a groove along the buccal, lingual, mesial and distal root surfaces with a tungsten carbide flat fissure bur. The tooth root was positioned in the small custom made vice with the mesial surface facing upwards. The tooth root was split by gently tapping the head of a screwdriver (flame sterilised) into the groove. If the root canals were not visible after splitting in the mesiodistal direction, tooth pieces were rotated 90° and split again using the pre-made buccolingual groove. Given the curvature of the roots, variations in the isthmi anatomy and the way the tooth roots split, it was not always possible to show the entire length of each canal (Figure 9).

Figure 9. Longitudinally split tooth root (Group 4: LAI 0.5 W) prepared for SEM imaging. One canal (right of picture) was split down the central lumen thereby allowing the entire length of the root canal to be visualised. The other canal (left of picture) did not split evenly.

The split root pieces were placed in SEM fixative for at least 24 hours before further processing. The split roots were then washed in PBS + 4% sucrose solution (5 min) and dehydrated by immersion in serial dilutions of ethanol (70% - 15 min; 90% - 15 min; 95% - 15 min; 100% - 3 x 10 min), critical point dried, mounted on SEM preparation slides and coated with platinum. The disks were then viewed with a field emission SEM (Phillips XL30, Eindhoven, Netherlands) to visualise if qualitative differences existed between treatment groups.

2.29 Statistics

The statistical software used for all calculations is SAS 9.3 (SAS Institute Inc., Cary, NC, USA). To determine if mean CFU/mL (culturing method) or mean cells/mL (qPCR method) are significantly different across treatment groups, linear mixed-effects models have been used. To account for clustering on flow cell, flow cell unit has been included as random effects whilst treatment group and confounders (for the adjusted models) are included as fixed effects.

The assumptions of a linear regression model were not upheld using outcome variables CFU/mL and cells/mL because these variables were very right skewed. Therefore the logarithms of CFU/mL and cells/mL were regressed against treatment (and confounders) and the assumptions of linear regression were found to be upheld. The estimates were then exponentiated to give geometric means and exponentiated 95% confidence intervals (CI). Comparison and Global P-values are also presented (Table 5 to Table 8). A P-value ≤ 0.05 is considered statistically significant.

3 Results

3.1 Primer optimisation

The qPCR forward and reverse universal primers amplified all stock DNA solutions (Appendices 6.3). The order of amplification, as represented by the average Ct values corresponded to the theoretically determined 16S rRNA gene copy number (Appendices 6.4). The 250(f):250(r) nanomolar primer concentrations were selected and used in all subsequent qPCR assays because this primer combination had the lowest Ct value and potential to form primer-dimer artefacts compared to the other primer concentrations (Appendices 6.5 and 6.6). The melt curve profile showed well defined unique peaks (Appendices 6.7).

3.2 Standard curve analysis

The slope of the standard curve, correlation coefficient (R^2) and amplification efficiency (E) for the mixed species standard curve assay were: y= -3.6025x + 32.637, 0.998, 90%, respectively (Appendices 6.8). These values are within an acceptable range (Dorak, 2006). The number of cycles necessary to amplify all serially diluted 16S rRNA DNA standards ranged from 6-28 (Appendices 6.9). Standards 10^{-6} and 10^{-7} were excluded from the standard curve because the Ct values and amplification profiles could not be clearly distinguished from the no template controls that formed primer-dimer artefacts. The standard curve was constructed using standards 10^{0} to 10^{-5} (i.e. 1.55 X 10^{7} to 1.55 X 10^{2} gene copies). The amplification profile (Appendices 6.10) was acceptable and the dissociation profile for these standards showed well-defined peaks (Appendices 6.11). The lower limit of detection was determined to be approximately 150 16S rRNA gene copies or 35 bacterial cells.

3.3 Treatment group 16S rRNA gene copy number

The slope of the standard curve was used to convert the standardised Ct values to 16S rRNA gene copy number using the formula $y = -3.6025x + 32.637$ = [16S rRNA gene copy number = $10^{(S.Ct - 32.637) / -3.6025)}$] where y is the standardised Ct number and x the $\log_{10}$ 16S rRNA gene copy number.

3.4 Flow cell analysis

Four flow cells (A,B,C,D) were used in this study to grow the mature mixed species biofilm. Statistical analysis was only performed on 66 roots out of the 72 roots from flow cells A, B and C (Appendices 6.12, 6.13 and 6.14). Six roots from flow cell A, B and C were excluded from statistical analysis: Two roots from flow cell A and one root from flow cell C were excluded from analysis because of processing errors and one root from each of the three flow cells A, B and C was used for SEM analysis. All culturing and qPCR results from flow cell D were excluded from statistical analysis because visual assessment of the nutrient plates revealed the presence of a contaminant organism (Appendices 6.15).

3.5 Descriptive statistics

Table 3 and Table 4 gives the median, first quartile, third quartile and sample size of each treatment group and method of quantifying bacteria (culturing (CFU/mL) and qPCR (cells/mL)). Mean and standard deviation are not presented due to the extremely right-skewed nature of the culturing and qPCR variables. Percentage kill (culturing data) is included in Table 3, with No Tx as the control. Percentage kill was calculated by: 100 X (control median – treatment median)/control median. Percentage bioburden reduction (qPCR data) is included in Table 4, with No Tx as the control. Percentage bioburden reduction was calculated by: 100 X (control median – treatment median)/control median.

Method (culturing)	Treatment group	Median	First quartile	Third quartile	Sample size	Percentage kill [1]
CFU/mL	No Tx	1.29E+04	6.18E+03	6.30E+04	5	0.0%
CFU/mL	Saline SI	2.25E+03	4.45E+02	1.92E+04	15	82.5%
CFU/mL	LAI 0.75 W	6.05E+02	0.00E+00	1.06E+03	10	95.3%
CFU/mL	UAI	1.75E+02	1.00E+01	5.25E+02	18	98.6%
CFU/mL	LAI 0.5 W	8.00E+01	3.50E+01	1.45E+02	18	99.4%

Table 3. Descriptive statistics and percentage kill for each treatment group as quantified using culturing methods (CFU/mL).
[1] No Tx median is considered as the control median.

Method (qPCR)	Treatment group	Median	First quartile	Third quartile	Sample size	Percentage bioburden reduction [1]
Cells/mL	No Tx	9.94E+06	7.15E+06	2.06E+07	5	0.0%
Cells/mL	Saline SI	3.05E+06	8.39E+05	5.43E+06	15	69.3%
Cells/mL	LAI 0.75 W	9.33E+05	6.50E+05	1.34E+06	10	90.6%
Cells/mL	UAI	6.42E+05	1.93E+05	1.19E+06	18	93.5%
Cells/mL	LAI 0.5 W	5.47E+05	2.05E+05	1.50E+06	18	94.5%

Table 4. Descriptive statistics and percentage bioburden reduction for each treatment group as quantified using qPCR methods (cells/mL).
[1] No Tx median is considered as the control median.

3.6 Linear mixed-effects models

3.6.1 Model 1: Unadjusted linear mixed-effects model of treatment group versus CFU/mL

The first linear mixed-effects model was unadjusted, with the predictor being treatment group, the outcome being logarithm of CFU/mL, and random effects being flow cell unit. A statistically significant association was found between CFU/mL and treatment group (Global P<0.0001) (Table 5).

3.6.1.1 Analysis of treatment groups

When post-hoc comparisons were performed, statistically significant differences were found between mean values of CFU/mL for No Tx and UAI (P<0.0001), No Tx and LAI 0.5 W (P<0.0001) and No Tx and LAI 0.75 W (P=0.0006) and also between Saline SI treatment and UAI (P<0.0001), Saline SI treatment and LAI 0.5 W (P<0.0001) and Saline SI treatment and LAI 0.75 W (P=0.0158). For example, teeth in the No Tx group had a mean CFU/mL value 490 units higher than teeth in the UAI group (exponentiated 95% CI: 39, 6140). No statistically significant differences were found between mean values of No Tx and Saline SI treatment, or between treatment groups UAI, LAI 0.5 W or LAI 0.75 W.

Method	Treatment group: comparison	Treatment group: reference	Geometric mean (exponentiated 95% CI)	Comparison P-value	Global treatment group P-value
CFU/mL	No Tx	Saline SI	10 (1, 129)	0.0706	<0.0001
CFU/mL	No Tx	UAI	490 (39, 6140)	<0.0001	
CFU/mL	No Tx	LAI 0.5 W	704 (56, 8822)	<0.0001	
CFU/mL	No Tx	LAI 0.75 W	140 (9, 2104)	0.0006	
CFU/mL	Saline SI	UAI	48 (8, 271)	<0.0001	
CFU/mL	Saline SI	LAI 0.5 W	69 (12, 390)	<0.0001	
CFU/mL	Saline SI	LAI 0.75 W	14 (2, 112)	0.0158	
CFU/mL	UAI	LAI 0.5 W	1 (0, 7)	0.6573	
CFU/mL	LAI 0.75 W	UAI	4 (0, 31)	0.2521	
CFU/mL	LAI 0.75 W	LAI 0.5 W	5 (1, 44)	0.1413	

Table 5. Model 1: Unadjusted linear mixed-effects model of treatment group versus CFU/mL.

3.6.2 Model 2: Adjusted linear mixed-effects model of treatment group versus CFU/mL

The second linear mixed-effects model was adjusted, with the predictor being treatment group, the outcome being logarithm of CFU/mL, confounders being position within flow cell, foramen type and curvature group, and random effects being flow cell unit. Position in flow cell (P=0.0612) and foramen type (P=0.2255) were not found to be statistically significant confounders whereas curvature type (P =0.0426) was found to be statistically significant. When all three confounders were added to the linear mixed-effects model, there was still a statistically significant association between treatment group and CFU/mL (P=0.0008) (Table 6).

3.6.2.1 Analysis of treatment groups

When post-hoc comparisons were performed, statistically significant differences were found between mean values of CFU/mL for No Tx and all other treatment groups, between Saline SI treatment and UAI (P=0.0146), Saline SI treatment and LAI 0.5 W (P=0.0031) and also between LAI 0.5 W and LAI 0.75 W (P=0.0209). For example, teeth in the Saline SI treatment group had a mean CFU/mL value 150 units higher than teeth in the LAI 0.5 W group (exponentiated 95% CI: 6, 3649). No statistically significant differences were found between mean values of Saline SI treatment and LAI 0.75 W or between UAI and LAI 0.5 W or LAI 0.75 W.

3.6.2.2 Analysis of position in flow cell, foramen type and curvature type

Global P-values indicated that position in flow cell and foramen type were not significantly associated with CFU/mL; therefore no further analysis was necessary. When post-hoc comparisons were performed for curvature group, no statistically significant differences were found between mean values of CFU/mL for curvature groups 1 (mildly curved) and 2 (moderately curved) or between curvature groups 2 and 3 (severely curved). A statistically significant difference was however found between mean values of CFU/mL for curvature groups 3 (severely curved) and 1 (mildly curved) (P=0.0183). Severely curved teeth had a mean CFU/mL value 14 units higher than mildly curved teeth, controlling for treatment type, foramen type and position in flow cell (exponentiated 95% CI: 1.62, 129).

Method	Treatment group: comparison	Treatment group: reference	Geometric mean (exponentiated 95% CI)	Comparison P-value	Global treatment group P-value
CFU/mL	No Tx	Saline SI	19 (1, 495)	0.0723	0.0008
CFU/mL	No Tx	UAI	730 (23, 23441)	0.0005	
CFU/mL	No Tx	LAI 0.5 W	2890 (101, 82988)	<0.0001	
CFU/mL	No Tx	LAI 0.75 W	184 (7, 5118)	0.0031	
CFU/mL	Saline SI	UAI	38 (2, 665)	0.0146	
CFU/mL	Saline SI	LAI 0.5 W	150 (6, 3649)	0.0031	
CFU/mL	Saline SI	LAI 0.75 W	10 (0, 273)	0.1815	
CFU/mL	UAI	LAI 0.5 W	4 (1, 29)	0.169	
CFU/mL	LAI 0.75 W	UAI	4 (0, 58)	0.3019	
CFU/mL	LAI 0.75 W	LAI 0.5 W	16 (2, 159)	0.0209	
CFU/mL	Curvature 2	Curvature 1	0.21 (0.71, 13.3)	0.1295	0.0426
CFU/mL	Curvature 3	Curvature 1	14.43 (1.62, 129)	0.0183	
CFU/mL	Curvature 3	Curvature 2	4.70 (0.49, 45.21)	0.1735	

Table 6. Model 2: Adjusted linear mixed-effects model of treatment group versus CFU/mL.
Adjusted for position within flow cell (P-value=0.0612), foramen type (P-value=0.2255) and curvature group (P-value=0.0426).

3.6.3 Model 3: Unadjusted linear mixed-effects model of treatment group versus cells/mL

The third linear mixed-effects model was unadjusted, with the predictor being treatment group, the outcome being logarithm of cells/mL, and random effects being flow cell unit.

A statistically significant association was found between logarithm of cells/mL and treatment group (Global P<0.0001) (Table 7).

3.6.3.1 Analysis of treatment groups

When post-hoc comparisons were performed, statistically significant differences were found between mean values of cells/mL for No Tx and all other treatment groups, and between Saline SI treatment and UAI (P=0.0041), Saline SI treatment and LAI 0.5 W (P=0.0125) and also between Saline SI treatment and LAI 0.75 W (P=0.007). For example, teeth in the Saline SI treatment group had a mean CFU/mL value 3 units higher than teeth in the UAI group (exponentiated 95% CI: 1, 7). No statistically significant differences were found between treatment groups UAI, LAI 0.5 W and LAI 0.75 W.

Method	Treatment group: comparison	Treatment group: reference	Geometric mean (exponentiated 95% CI)	Comparison P-value	Global treatment group P-value
Cells/mL	No Tx	Saline SI	3.07 (1, 12)	0.0121	<0.0001
Cells/mL	No Tx	UAI	13 (4, 39)	<0.0001	
Cells/mL	No Tx	LAI 0.5 W	11 (4, 33)	<0.0001	
Cells/mL	No Tx	LAI 0.75 W	15 (5, 50)	<0.0001	
Cells/mL	Saline SI	UAI	3 (1, 7)	0.0041	
Cells/mL	Saline SI	LAI 0.5 W	3 (1, 6)	0.0125	
Cells/mL	Saline SI	LAI 0.75 W	4 (1, 10)	0.007	
Cells/mL	UAI	LAI 0.5 W	1 (0, 2)	0.6565	
Cells/mL	LAI 0.75 W	UAI	1 (0, 2)	0.7129	
Cells/mL	LAI 0.75 W	LAI 0.5 W	1 (0, 2)	0.5023	

Table 7. Model 3: Unadjusted linear mixed-effects model of treatment group versus cells/mL.

3.6.4 Model 4: Adjusted linear mixed-effects model of treatment group versus cells/mL

The fourth linear mixed-effects model was adjusted, with the predictor being treatment group, the outcome being logarithm of cells/mL, confounders being position within flow cell, foramen type and curvature group, and random effects being flow cell unit. Position in flow cell (Global P-value=0.3672), foramen type (Global P-value=0.5587) and curvature type (Global P-value=0.7406) were not found to be statistically significant confounders. Therefore, when the three confounders were added to the linear mixed-effects model,

treatment group was still statistically significantly associated with cells/mL (Global P=0.0154) (Table 8).

3.6.4.1 Analysis of treatment groups

When post-hoc comparisons were performed, statistically significant differences were found between mean values of cells/mL for No Tx and all other treatment groups. For example, teeth in the No Tx group had a mean cells/mL value 11 units higher than teeth in the LAI 0.5 W group, controlling for position in flow cell, curvature group and foramen type (exponentiated 95% CI: 2,55) (P=0.0065). No statistically significant differences were found between Saline SI treatment and treatment groups UAI, LAI 0.5 W or LAI 0.75 W. No statistically significant differences were found between treatment groups UAI, LAI 0.5 W and LAI 0.75 W.

3.6.4.2 Analysis of position in flow cell, foramen type and curvature type

Global P-values indicated that position in flow cell, foramen type and curvature type did not have a statistically significant association with cells/mL.

Method	Treatment group: comparison	Treatment group: reference	Geometric mean (exponentiated 95% CI)	Comparison P-value	Global treatment group P-value
Cells/mL	No Tx	Saline SI	10 (2, 47)	0.006	0.0154
Cells/mL	No Tx	UAI	21 (4, 119)	0.0009	
Cells/mL	No Tx	LAI 0.5 W	11 (2, 55)	0.0065	
Cells/mL	No Tx	LAI 0.75 W	9 (2, 46)	0.009	
Cells/mL	Saline SI	UAI	2 (1, 9)	0.2645	
Cells/mL	Saline SI	LAI 0.5 W	1 (0, 5)	0.9246	
Cells/mL	Saline SI	LAI 0.75 W	1 (0, 5)	0.9368	
Cells/mL	UAI	LAI 0.5 W	0 (0, 1)	0.1442	
Cells/mL	LAI 0.75 W	UAI	2 (1, 9)	0.2172	
Cells/mL	LAI 0.75 W	LAI 0.5 W	1 (0, 4)	0.8154	
Cells/mL	Curvature 3	Curvature 2	1.20 (0.39, 3.67)	0.7397	0.7406
Cells/mL	Curvature 3	Curvature 1	1.45 (0.49, 4.24)	0.4906	
Cells/mL	Curvature 2	Curvature 1	1.20 (0.59, 2.46)	0.6029	

Table 8. Model 4: Adjusted linear mixed-effects model of treatment group versus cells/mL.
Adjusted for position in flow cell (P-value=0.3672), curvature group (P-value=0.7406) and foramen type (P-value=0.5587)

3.7 SEM – qualitative analysis

3.7.1 No treatment

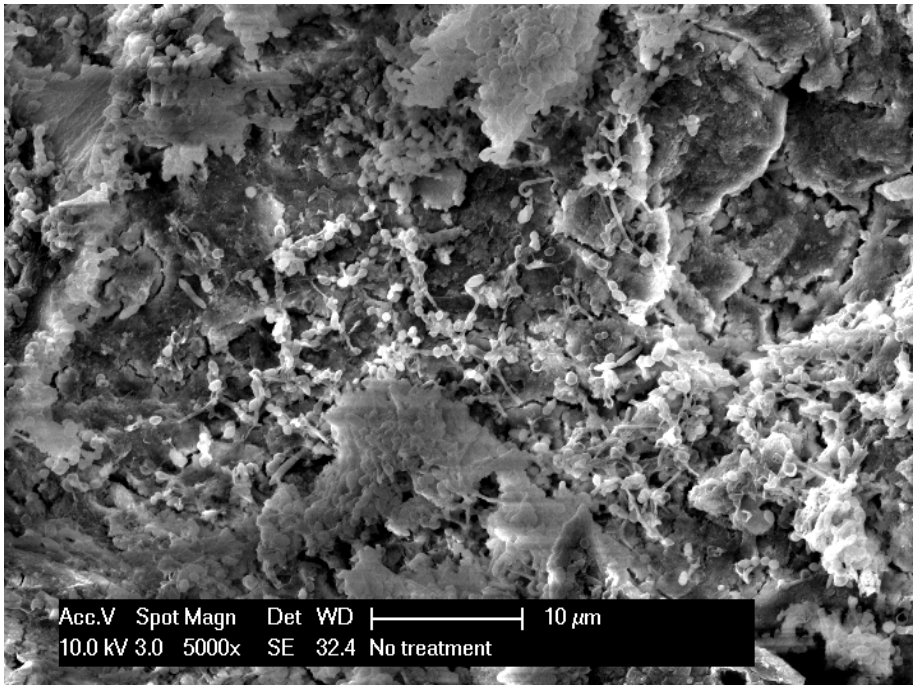

Figure 10. SEM image of the 4 week biofilm within the root canal of the no treatment group (5,000 x magnification).
A thick, multilayered biofilm is evident.

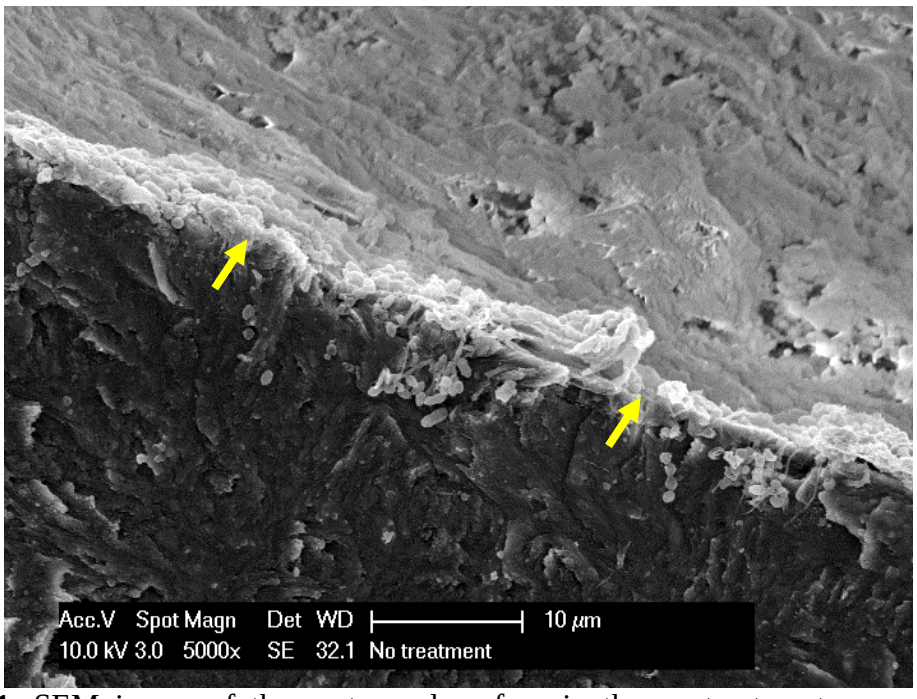

Figure 11. SEM image of the root canal surface in the no treatment group (5,000 x magnification).
A thick biofilm is present. It covers the entire main root canal surface (yellow arrows).

3.7.2 Saline standard irrigation

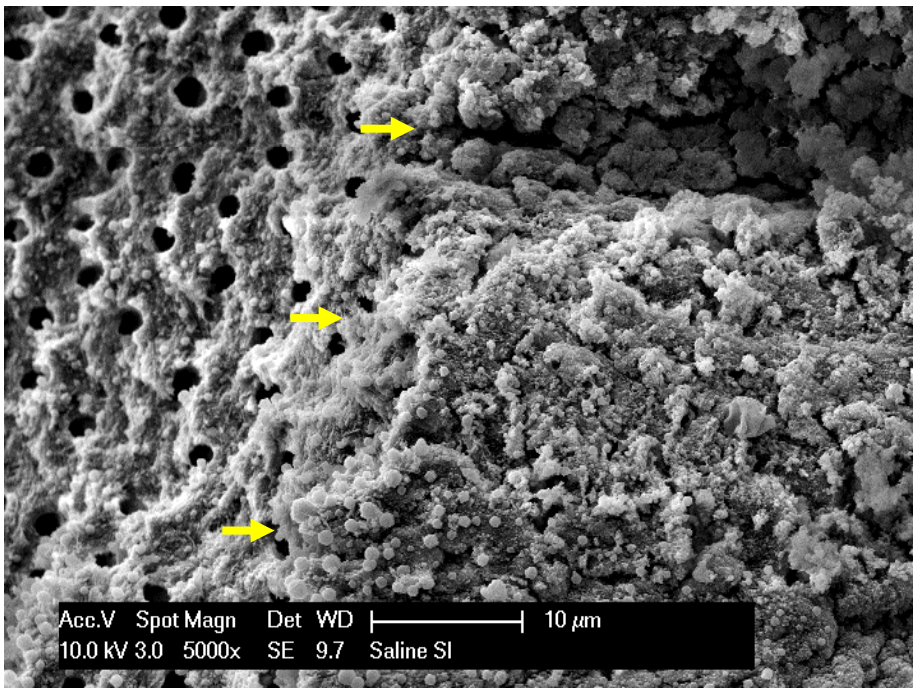

Figure 12. SEM image of the root canal surface following mechanical preparation and saline standard irrigation (5,000 x magnification).
A thick mixture of smear layer and biofilm line the main root canal surface (right of the yellow arrows). Dentine tubules are visible (left of the yellow arrows).

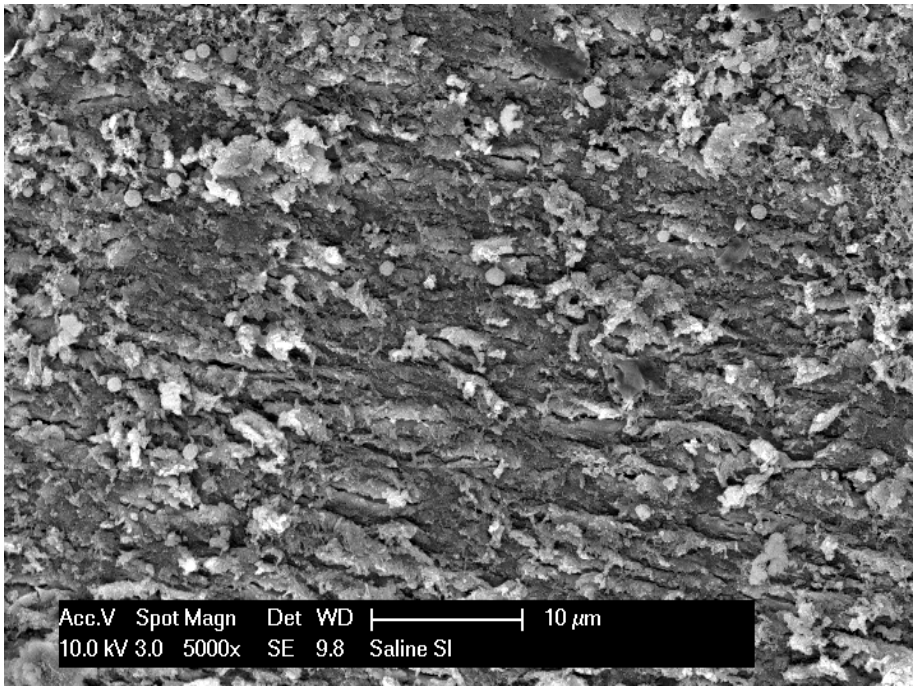

Figure 13. SEM image of the root canal surface following mechanical preparation and saline standard irrigation (5,000 x magnification).
Biofilm and individual cocci are intermingled with a smear layer that completely covers the root canal surface.

3.7.3 Ultrasonic activated irrigation

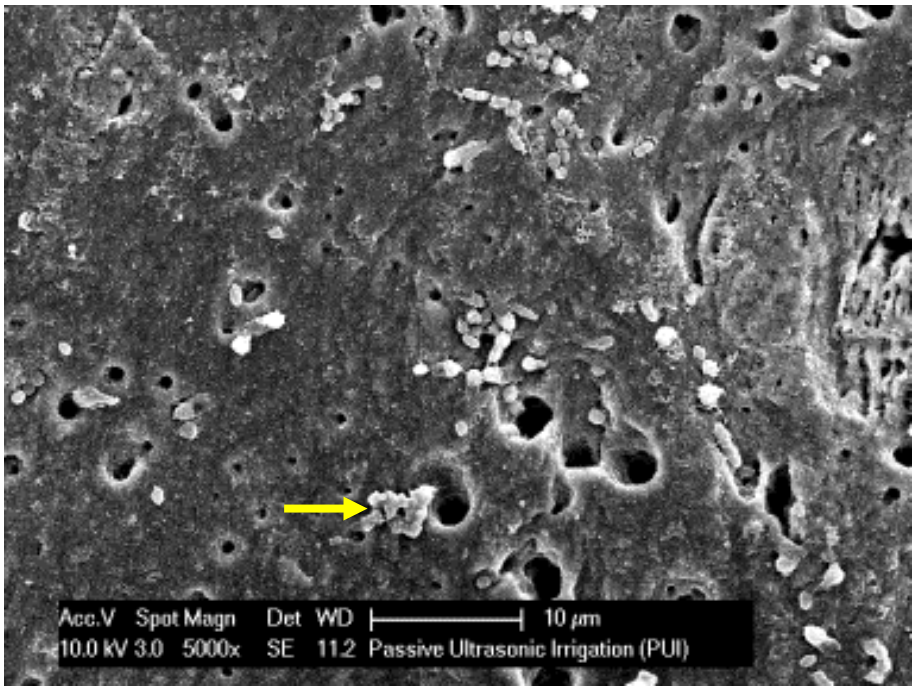

Figure 14. SEM image of the root canal surface following chemomechanical preparation and ultrasonic activated irrigation with 4% NaOCl and 15% EDTAC (5,000 x magnification).
No biofilm is present although individual cocci and co-aggregated bacteria (yellow arrow) are visible on the dentine surface.

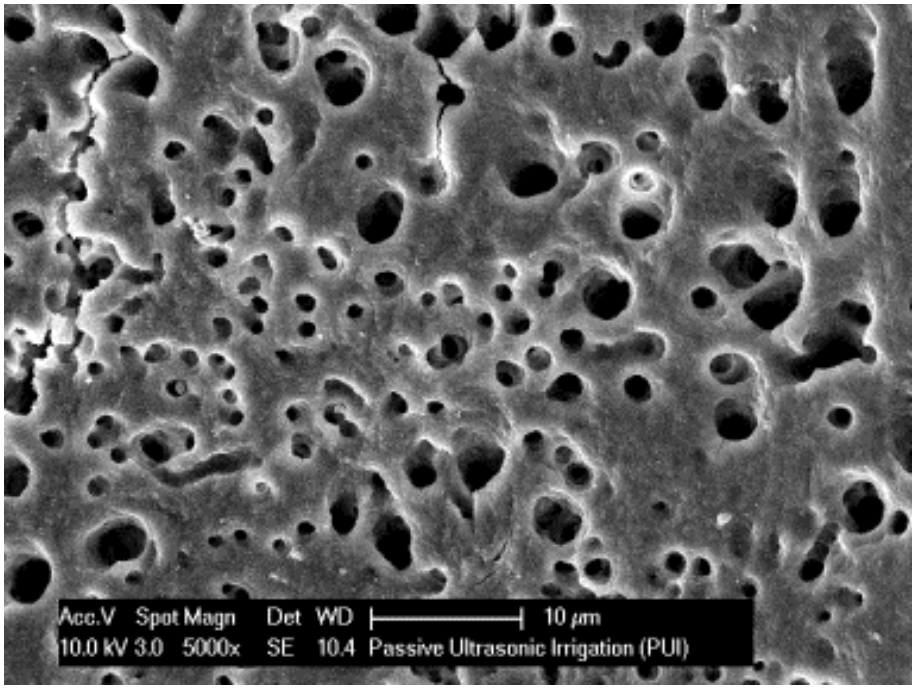

Figure 15. SEM image of the root canal surface following chemomechanical preparation and ultrasonic activated irrigation with 4% NaOCl and 15% EDTAC (5,000 x magnification).
No bacteria are visible. The dentine tubules are patent and irregular in size.

3.7.4 Laser activated irrigation 0.5 W

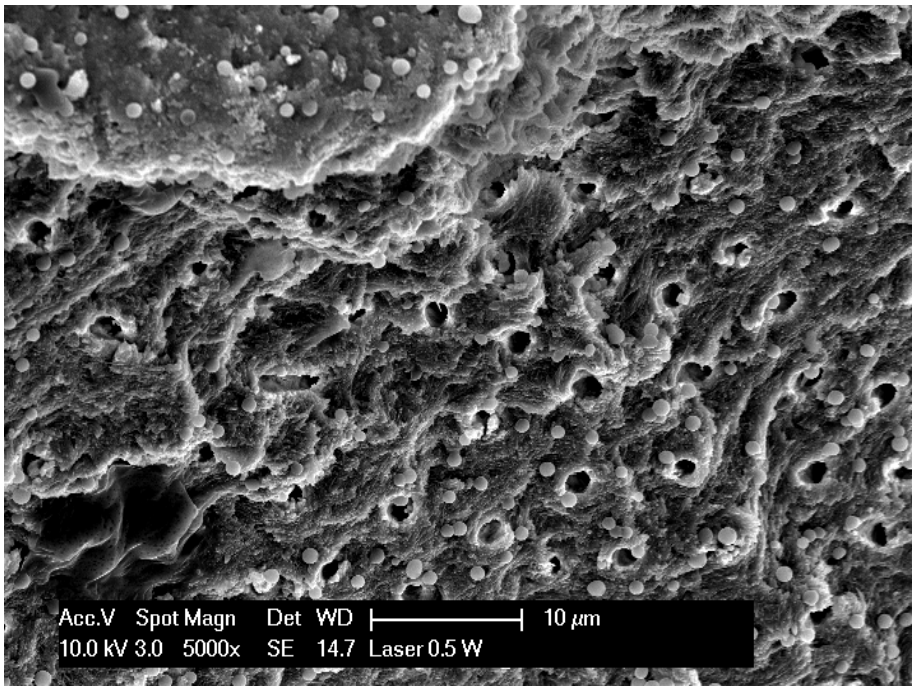

Figure 16. SEM image of the root canal surface following chemomechanical preparation and laser activated irrigation 0.5 W with 4% NaOCl and 15% EDTAC (5,000 x magnification).
Individual cocci are visible on the dentine surface.

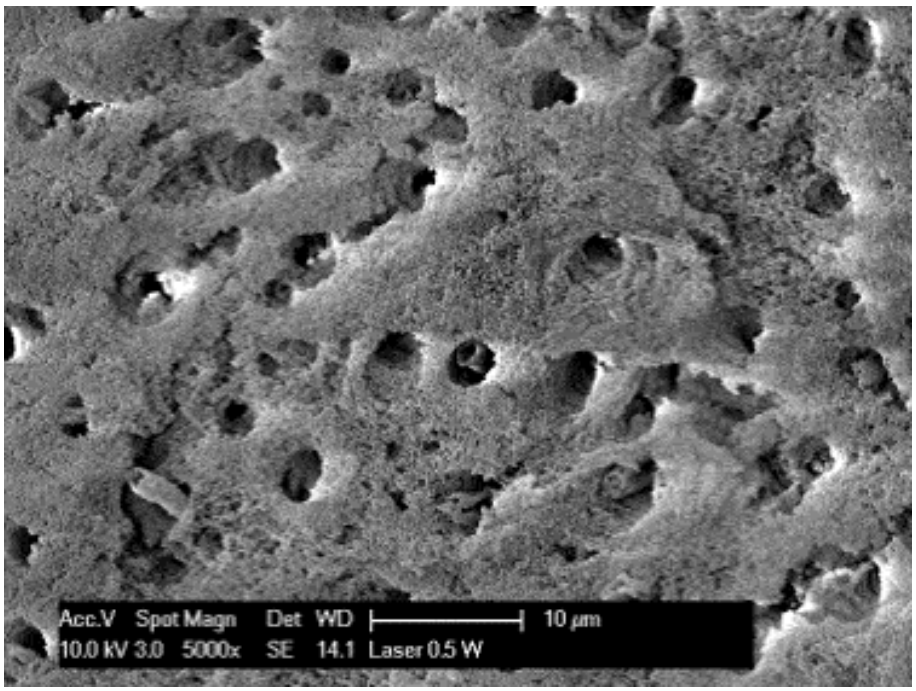

Figure 17. SEM image of the root canal surface following chemomechanical preparation and laser activated irrigation 0.5 W with 4% NaOCl and 15% EDTAC (5,000 x magnification).
The dentine tubules are visible although the dentine surface appears eroded. No bacteria are visible.

3.7.5 Laser activated irrigation 0.75 W

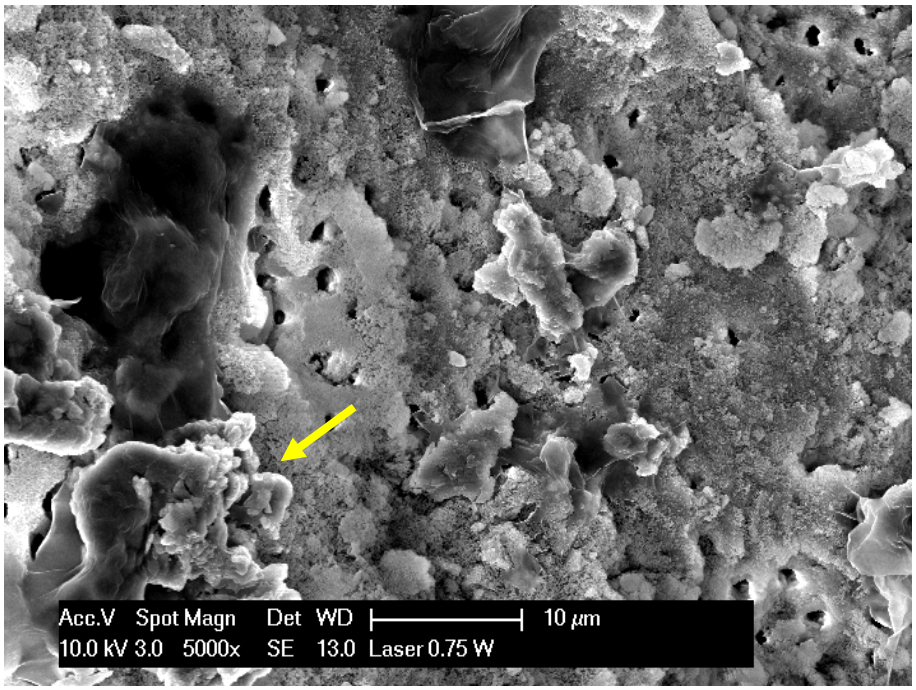

Figure 18. SEM image of the root canal surface following chemomechanical preparation and laser activated irrigation 0.75 W with 4% NaOCl and 15% EDTAC (5,000 x magnification).
The dentine surface is covered with biofilm remnants (yellow arrow) and a smear layer. Only a few dentine tubules are visible.

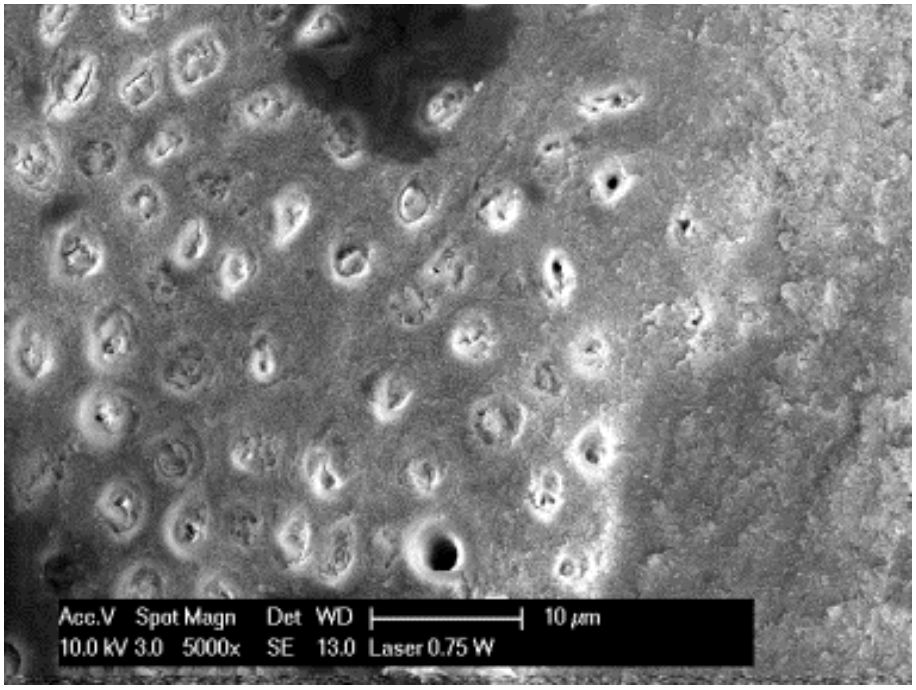

Figure 19. SEM image of the root canal surface following chemomechanical preparation and laser activated irrigation 0.75 W with 4% NaOCl and 15% EDTAC (5,000 x magnification).
No bacteria are visible and the majority of the dentine tubules appear sclerosed.

SEM analysis of root canals showed qualitative differences between the treatment groups. The root canal from the No Tx group showed the presence of a continuous thick mature mixed species biofilm with clearly discernible cocci and rod shaped bacteria. The bacteria were attached to the dentine and to neighbouring cells by a thick extra-cellular matrix. The Saline SI group showed a substantial reduction in the quantity of biofilm although most of the root canal surfaces were covered by a thick smear layer composed of biofilm and extracellular matrix remnants (Figure 13). Patent dentine tubules were rarely seen.

The UAI and LAI (0.5 W and 0.75 W) groups showed a significant reduction in the quantity of biofilm compared to the No Tx and Saline SI groups. The root canals from the UAI and LAI groups (0.5 W and 0.75 W) showed no qualitative differences. Most of the root canal surface of the UAI and LAI groups (0.5 W and 0.75 W) were free from biofilm, debris and smear layer although individual cocci (Figure 16), co-aggregated bacteria (Figure 14) or small areas of biofilm could be seen (Figure 18). Significant erosion of the dentine surface (Figure 17) was observed in many areas of the canals with the UAI and LAI (0.5 W and 0.75 W) groups.

4 Discussion

4.1 Flow cell and biofilm growth

A flow cell model developed by (Seet *et al.*, 2012) was used to grow a multi-species *in vitro* biofilm within the root canal of lower molar mesial roots. SEM analysis showed that a mature biofilm lined the root canal walls. The protocol for growing the multispecies biofilm was based on the work of Yap *et al.* (2014). They grew the biofilm on dentine discs whereas the present study used the more complex root canal anatomy of human lower molar mesial roots which more closely mimics the *in vivo* environment.

A multi-species biofilm containing *E. faecalis*, *S. sanguinis* and *F. nucleatum* was used in this study because it goes some way towards replicating the polymicrobial intraradicular bacterial infection compared to axenic biofilms used in many previous studies. These three species were chosen for a number of reasons. Firstly, initial endodontic treatment eliminates most Gram-negative bacteria (except for some anaerobic rods such as *F. nucleatum*) whereas Gram-positive bacteria (e.g. *S. sanguinis* and *E. faecalis*) are significantly more resistant to treatment procedures (Siqueira *et al.*, 2009). Secondly, all three species have been commonly detected in post treatment failures (Siqueira & Rôças, 2004; Fujii *et al.*, 2009; Schirrmeister *et al.*, 2009; Murad *et al.*, 2014). Finally, *S. sanguinis* (Lancy *et al.*, 1983) and *E. faecalis* (Johnson *et al.*, 2006) have been shown to coaggregate with *F. nucleatum* and all three have been shown to grow *in-vivo* as either mono (Chew *et al.*, 2012), dual (Bryce *et al.*, 2009) or multi-species biofilms (Yap *et al.*, 2014).

4.2 Bacterial penetration of dentine tubules

The present study showed that cocci shaped bacteria could invade the dentine tubules. Both *S. sanguinis* and *E. faecalis* are able to invade the dentine tubules to varying extents (Akpata & Blechman, 1982). With three weeks incubation, *S. sanguinis* can penetrate approximately one third the thickness of the pulpal dentine wall whereas *E. faecalis* is able to penetrate to deeper levels within the same time period (Akpata & Blechman, 1982). *E. faecalis* has been reported to invade to depths greater than 600 µm (Parmar *et al.*, 2011; Ran *et al.*, 2014). Bacterial penetration of dentine tubules is deepest in the coronal and

middle sections whereas significantly lower depths occur in the apical region (Akpata & Blechman, 1982; Love, 1996).

In-vitro studies show that penetration depths are significantly affected by nutritional conditions and culturing times (Love & Jenkinson, 2002; Parmar *et al.,* 2011). Ran *et al.* (2014) recently showed penetration depths were significantly affected by the availability of glucose and alkalinity of the nutrient medium. When no glucose was available, dentine tubule penetration reduced by approximately 60% compared to when grown in tryptic soy broth which contains glucose. Increasing the alkalinity from a pH of 7 to pH 10 caused a reduction in dentine tubule penetration by approximately 80%. Penetration is also affected by the culturing time (Schäfer & Bössmann, 2005). The present study used a 3-4 week incubation period which has been shown to be long enough for substantial tubule invasion (Zapata *et al.,* 2008). Dentine tubule penetration may be limited by the availability of space, nutrient supply and oxygen levels (Ran *et al.*, 2014).

4.3 SEM sectioning and bacterial recovery

Tooth roots were longitudinally sectioned in the present study to qualitatively assess the efficacy of removing biofilm from the main canal walls using different treatment protocols. Longitudinal sectioning prevented SEM analysis of the isthmus region. It is assumed that bacteria most likely colonised the isthmi region of the lower molar mesial roots. Histobacteriological studies by (Nair *et al.,* 2005) and Vera *et al.* (2012) showed that bacteria readily colonise the isthmi region of lower molar mesial roots and that traditional root canal treatment, with or without the use of an intraradicular medicament, is ineffective in removing all bacteria from this region. The present study used a tooth crushing device prior to bacteria sampling. This should have greatly improved the detection of bacteria residing within the complex anatomical features, including the isthmus region of the root canal system compared to paper point sampling which only samples bacteria within the main canal (Akpata, 1976; Sathorn *et al.*, 2007).

It is unknown whether the total microbe viability was reduced by crushing the roots, but this was consistent across all treatment groups. A pilot study, if performed, may have helped address this issue. It was assumed that *E. faecalis* and *S. sanguinis* would survive tooth crushing given that they are both Gram-positive bactertia that are commonly found in

harsh environment such as an infected root filled tooth (Rôças & Siqueira, 2012).

4.4 Contamination of flow cell D and flow cell layout limitations

The treatment results from flow cell D were eliminated from statistical analysis because visual assessment of the nutrient plates revealed that many of the plates were contaminated with an unknown bacteria. The flow cell most likely became contaminated while exchanging empty nutrient media bottles with fresh nutrient media. Efforts were made to try and minimise contamination by using aseptic measures. Perhaps if the apparatus was setup in an exhaust chamber then contamination may not have occurred.

It is possible that bias may exist between treatment groups in the different flow cells as no flow cell to flow cell comparison of the enumerated CFU/mL and cells/mL data was performed. This might be important considering the uneven distribution of treatment conditions between flow cells. In retrospect, it would have been simpler to compare treatment effects on roots from the one flow cell layout, with each flow cell being an experimental replicate. If flow cell contamination occurred then exlusion from analysis would have less statistical significance on the analysis of the roots from the uncontaminated flow cells.

Despite the loss of flow cell D samples, each treatment group had adequate numbers for the statistical analysis to remain valid. Loss of data from flow cell D meant that there was no inter-flow cell replicate of tooth roots subject to LAI 0.75 W. The data obtained from flow cell B may be representative of the level of biofilm in flow cell B, however it is not possible to be confident of this.

Two samples from flow cell D were used for SEM analysis of the LAI 0.5 W and 0.75 W treatment groups. Although not ideal, the contaminated roots were still used because SEM analysis provides qualitative evidence of the amount of biofilm remaining on the dentine surface after treatment. NaOCl is a powerful antibacterial solution (Zehnder, 2006). It is unlikely that the contaminent survived treatment with 4% NaOCl any more readily than either *E. faecalis* and *S. sanguinis*. The contaminent however may have been better suited to growing rapidly in an aerobic environment (*e.g.* bacillus) compared to either *E. faecalis* and *S. sanguinis* and was therefore able to overgrow these bacteria on the

nutrient plate. Comparison of the SEM images showed no differences between the UAI and LAI (0.5 W and 0.75 W) treatment groups which is in agreement with quantitiative analysis of samples from the uncontaminated flow cells (A-C).

The SEM image representing the LAI 0.75 W treatment group shows that the canals were sclerosed. This was not reported for roots of the other treatment group. The sclerosed nature of the canals is most likely due to the random selection of teeth used for this SEM analaysis rather than being related to the LAI 0.75 W treatment group protocol.

4.5 Quantification limitations

Culturing was used to determine the efficacy of the treatment protocols. It is currently unknown how to selectively culture and accurately quantify *F. nucleatum* from a mixed species containing *E. faecalis, S. sanguinis* and *F. nucleatum*. A pilot study by Yap *et al.* (2014) revealed that *F. nucleatum* was overgrown by *E. faecalis* and/or *S. sanguinis* when cultured in an anaerobic environment. *F. nucleatum* is a strict anaerobic organism and will not grow in an aerobic environment (Bolstad *et al.*, 1996). For these reasons, only *E. faecalis* and *S. sanguinis* bacteria that survived treatment were quantified in this study using traditional culturing methods. Therefore the amount of bacteria that survived treatment is most likely underestimated. This predicament is not unique to the present study. Molecular techniques have confirmed the polymicrobial nature of endodontic infections and shown that the number of bacteria and species richness is far greater than revealed by culturing based studies.

Quantitative PCR was restricted to the use of universal rDNA primers and not species-specific primers because the main focus of the present study was to examine and compare total bacterial load reduction for each treatment group.

In a pilot study, multiple species-specific primers were developed and tested in an attempt to precisely quantify the amount of *E. faecalis, S. sanguinis* and *F. nucleatum* in a mixed sample. Although *F. nucleatum* could be independantly amplified in the presence of both *E. faecalis* and *S. sanguinis* 16S rRNA gene copies, it was not possible to develop primers that could reliably amplify *E. faecalis* but not *S. sanguinis* in a mixed species solution.

The Streptococcus species and *E. faecalis* species-specific primer sets used in a recent study by (Rôças & Siqueira, 2012) were also investigated in a pilot study however specificity remained a problem. This is not unexpected given that *E. faecalis* and *S. sanguinis* share a high degree of similarity between their highly conserverd 16S rRNA genes. It is likely that species-specific primers could have been developed using genes other the the 16S rRNA gene however this was not possible within the financial and time constraints of the present study.

4.6 Laser power setting and activation time

Studies that have investigated bacteria reduction as a result of LAI have used different types of lasers, subablative energy levels, laser tip design and activation times. Comparing the outcomes of these studies is therefore difficult because of the heterogeneous study designs.

The power setting in the present study is higher than that used by other studies that have investigated bacteria reduction using the Photon-Initiated Photoacoustic Streaming (PIPS) protocol (Peters *et al.*, 2011; DiVito *et al.*, 2012; Pedullà *et al.*, 2012; Zhu *et al.*, 2013b; Olivi *et al.*, 2014). This protocol recommends that a Er:YAG laser be used on a power setting of approximately 0.3 W (20 mJ at 15 Hz or 50 mJ at 10 Hz) and that the PIPS radial firing tip be placed only in the pulp chamber that is filled with irrigating solution.

Increasing the activation time may increase bacteria reduction however no study has directly tested this parameter. As used in the present study, Seet *et al.* (2012) and Christo (2012) both used a Er,Cr:YSGG laser and end-firing laser tips (RFT 3, 17 mm laser tip). They laser activated the NaOCl for 15 seconds and then irrigated the canals with 1.25 mL of NaOCl for 15 seconds. The cycle was repeated four times. Seet *et al.* (2012) used a power setting of 0.25 W (12.5 mJ at 20 Hz) whereas Christo (2012) used a power setting of 0.5 W (25 mJ at 20 Hz). Both these studies showed that *E. faecalis* biofilm was effectively reduced from the straight root canal walls using LAI with 4% NaOCl.

Most studies using the PIPS protocol have used a total laser activation time of between 60 - 90 seconds (Peters *et al.*, 2011; Pedullà *et al.*, 2012; Zhu *et al.*, 2013b; Olivi *et al.*,

2014). Neelakantan *et al.* (2014) modified the PIPS protocol by increasing the power setting to power setting to 0.5 W (50 mJ at 10 Hz) which is similar to the present study. They also used a total activation time of 4 minutes which is significantly longer than the present study and all other LAI based research.

In the present study, after the establishment of a 4 week mixed species biofilm, the canals were chemomechanically prepared with endodontic files, 4% NaOCl and 15% EDTAC. A Er,Cr:YSGG laser with end-firing laser tip (RFT 3, 17 mm laser tip) was used at power settings of 0.5 W (25 mJ at 20 Hz) and 0.75 W (37.5 mJ at 20 Hz) to ensure that the irrigating solutions were sufficiently energised to maximise the flow of irrigant into the complex anatomical features that exist in lower molar mesial roots.

4.7 Clinical relevance of the initial preparation and the chemomechanical preparation of the teeth

The chemomechanical protocol used in this *ex-vivo* study was chosen because it represents a clinically relevant protocol that is routinely used to treat lower molar mesial roots with apical periodontitis. The lower molar mesial roots were initially prepared to 25/.04 1 mm beyond the apex to allow establishment of the biofilm. The canals were then mechanically enlarged up to an apical size and canal taper of 35/.06 to facilitate the placement of a fine 30-gauge irrigating needle to within 1 to 2 mm of the apex in mildly or moderately curved roots. Placement of the irrigating needle to within close proximity of the working length, without binding, has been shown to increase the flow of irrigant into this problematic area (Boutsioukis *et al.*, 2010a).

Four percent NaOCl was used in this study because most studies that have investigated LAI and bacteria reduction have used between 3 and 6% NaOCl. EDTAC was incorporated into the treatment protocol because EDTAC has been shown to remove the smear layer (Violich & Chandler, 2010). Removal of the smear layer improves the penetration of NaOCl into the dentine tubules to further reduce bacteria number (Byström & Sundqvist, 1985). In the present study 4% NaOCl was used during the mechanical preparation phase. Following this, UAI or LAI was used to activate 15% EDTAC before activating the 4% NaOCl. In comparison to standard syringe irrigation, smear layer removal may be improved when either ultrasonic (Andrabi *et al.*, 2014) or laser

(Lagemann *et al.*, 2014) energy is used to activate EDTA. It is not conclusively known if there is a major antibacterial difference between EDTA and EDTAC (Zehnder, 2006) and it is unlikely to significantly influence the results.

Ultrasonic activation of the irrigants was used as a supplementary protocol because it is relatively inexpensive, routinely used by endodontists (Dutner *et al.*, 2012) and has been shown to improve the effectiveness of the chemomechanical preparation procedure in lower molars (Burleson *et al.*, 2007) (Carver *et al.*, 2007). It can therefore be considered the "gold standard" by which to compare the efficacy of LAI.

It is not clear given the significant variations between methodologies and outcomes whether LAI is more effective than conventional syringe irrigation when high concentrations (3-6%) of NaOCl and fine 30-gauge needles are used. No study yet has chemomechanically prepared infected root canals before LAI treatment. Therefore it is currently unknown whether LAI is more or less effective than traditional chemomechanical procedures.

4.8 The efficacy of the treatment protocols as determined by culturing methods

All samples in the No Tx group were positive for bacteria. The median number of bacteria in the No Tx group was similar to *in vivo* studies that sampled bacteria from infected root canals before traditional root canal therapy was commenced (Byström & Sundqvist, 1981; 1985; Sjögren *et al.*, 1991; Peters *et al.*, 2002) (Vianna *et al.*, 2006). The No Tx group sample range was considerably lower than other studies. This is most likely due to the small sample size and also due to standardisation of the root length and canal taper before placement in the flow cell. The low range is ideal when trying to test the efficacy of one treatment protocol against another.

Statistically significant differences were found between mean values of CFU/mL for No Tx and all other treatment groups. This confirms that conventional instrumentation and irrigation techniques effectively reduce bacteria number from infected root canals when culturing methods are used (Shuping *et al.*, 2000; Card *et al.*, 2002; Tan & Messer, 2002; Rôças *et al.*, 2013).

Saline has minimal antibacterial properties, however irrigation with saline facilitates the removal of debris, planktonic microorganism and loosely attached biofilm from the canal via its flushing mechanism. The present study showed that mechanical preparation and standard irrigation using saline removes significant amounts of bacteria from the root canal system. This has also been shown by a number of other studies. Byström & Sundqvist (1981) showed a 100-1000 fold bacteria reduction in straight roots whereas Aydin *et al.* (2007) reported a 13-fold (98%) reduction in premolar canals when infected canals were mechanically enlarged with the use of saline as the irrigant.

In the present study, mechanical preparation and saline irrigation led to a 6-fold (83%) reduction in bacteria number although no roots were free from bacteria. In an *in-vivo* experiment, Dalton *et al.* (1998) reported a 96% (26-fold) reduction in bacteria number when the lower molar mesial roots were mechanically instrumented and irrigated with saline. Approximately 25% of the canals in their study were found to be free of bacteria. The efficacy of the instrumentation procedure in the present study appears to be much lower than that reported by (Dalton *et al.*, 1998) however there may be a reason for this. Dalton *et al.* (1998) used paper points to sample only bacteria in the main root canal whereas the present study crushed the roots after treatment. Crushing the roots after treatment most likely increased the number of bacteria in each sample (Akpata, 1976).

NaOCl is an excellent antibacterial solution (Berber *et al.*, 2006) and can improve the efficacy of the chemomechanical procedure (Shuping *et al.*, 2000). The present study confirms these findings by showing that bacterial numbers were significantly reduced when mechanical instrumentation was combined with 4% NaOCl and 15% EDTAC syringe irrigation and either UAI or LAI 0.5 W in comparison to saline. The improved efficacy is most likely due to both the inherent bactericidal properties of NaOCl and the improved irrigant flow dynamics (de Groot *et al.*, 2009; Matsumoto *et al.*, 2011; Boutsioukis *et al.*, 2014) created by the energy imparted to the irrigant via the ultrasonic and laser devices.

The importance of NaOCl in reducing bacterial numbers when combined with LAI, sonic activated irrigation or UAI was demonstrated by Seet *et al.* (2012) and Neelakantan *et al.* (2014). Seet *et al.* (2012) showed in a qualitative study, that laser (0.25 W) or sonic activation of 4% NaOCl was far more effective at removing bacteria compared to when

saline was used as the irrigant. Neelakantan *et al.* (2014) confirmed that bacteria reduction is significantly improved when NaOCl is either laser or ultrasonically activated compared to when saline is activated by these modalities.

In the present study, a significant amount of biofilm and infected dentine was removed from the main root canal as a result of the instrumentation procedure. The majority of the remaining bacteria may have been located in regions of the root canal system which are impervious to physical or chemical disruption by NaOCl when delivered via a syringe (Harrison *et al.*, 2010; Tay *et al.*, 2010). Mechanical instrumentation with 4% NaOCl and 15% EDTAC combined with LAI (0.75 W) was no more effective at removing bacteria than when saline was used as the irrigant. This is a surprising result and may have occurred because LAI (0.75 W) fluid dynamics did not improve the flow of 4% NaOCl into the deeper recesses of the root canal system.

LAI 0.5 W was more effective at removing bacteria than LAI 0.75 W. The reason for this may be due to the methodology used in the present study. A light chattering sound could be heard when 2 mm of the laser tip was fully immersed and activated within the irrigating solution. When the laser tip was in contact with the surface of the solution, the chattering sound increased in volume. Interestingly, the louder chattering sound could be heard when performing LAI at 0.75 W but not at 0.5 W. It was also noticed that after activation of the laser at 0.75 W, the solution in the canal was about 1-2 mm lower than the canal orifice whereas the irrigating solution was almost always flush with the canal orifice when the laser power was set to 0.5 W. An explanation might be that the increased laser power setting caused the irrigating solution to spray out of the canal because there was no coronal reservoir to stop this from occurring. By not having the tip of the laser fully immersed in irrigating solution, effective cavitation (Blanken & Verdaasdonk, 2007; Matsumoto *et al.*, 2011) or Photon-Initiated Photoacoustic Streaming (DiVito *et al.*, 2012) may have not occurred, thereby limiting both irrigant turbulence (Siqueira & Rôças, 2011) and the availability of free chlorine ions (Macedo *et al.*, 2010). This in turn would have reduced the efficacy of the LAI 0.75 W protocol.

The apparent loss of irrigant during laser activation is not unique to the present study. Neelakantan *et al.* (2014) and Zhu *et al.* (2013b) both followed the PIPS protocol (Peters *et al.*, 2011; Al Shahrani *et al.*, 2014; Neelakantan *et al.*, 2014; Olivi *et al.*, 2014) which

recommends the conical shaped laser tip be placed only in the pulp chamber. They also did not provide a continuous flow of irrigant into the pulp chamber during laser activation as recommended by the PIPS protocol. They both noticed that during laser activation, the pulp chamber became depleted of irrigant which needed to be refreshed. The reason for loss of irrigant in the pulp chamber has not been explained and requires further study to investigate why this is occurring.

The null hypothesis that there is no difference between LAI and UAI in eradicating a mixed species biofilm from the mesial roots of human mandibular molars when used as a supplementary approach to traditional chemomechanical methods was accepted when culturing methods were utilised. A significant difference was not shown between UAI and LAI 0.5 W or between UAI and LAI 0.75 W. These findings confirm the findings of others (Peters *et al.*, 2011; Neelakantan *et al.*, 2014) when reduction in bacteria number is used to compare the efficacy of the treatment protocols.

Peters *et al.* (2011) and Neelakantan *et al.* (2014) and the present study have the following variables in common: Firstly, three week or older, mature biofilms were established in human root canals before treatment using concentrations of 3% NaOCl or greater. Secondly, the types of lasers and power output (0.5 W) were also similar and thirdly, the efficacy of UAI and LAI (0.5 W) treatment protocols were assessed using culturing methods. Considerable methodological variations exist between the present study and the studies by Peters *et al.* (2011) and Neelakantan *et al.* (2014), hence direct comparison between studies needs to be done with caution. These methodological differences include variations in the bacteria species, complexity of the root canal system, mechanical and irrigating protocols, laser tip design and position in the canal, activation time, NaOCl contact time and volumes used. The microbial sampling methods and statistical analyses also differed.

Peters *et al.* (2011) used both negative cultures and bacteria reduction to determine treatment efficacy. When negative cultures were assessed they found LAI to have an increased antibacterial effect compared to UAI and syringe irrigation. The present study found negative cultures in only 4/18 (UAI), 3/18 (LAI 0.5 W) and 3/10 (LAI 0.75 W) treatment groups, however these culturing results were based on tooth crushing to liberate viable bacteria rather than the modified paper point sampling technique that was used by

Peters *et al.* (2011). The problem with using a binary outcome measure to assess treatment efficacy is that it ignores the bacteria load that remains within teeth that had positive culturing results.

Peters *et al.* (2011), like the present study, found no statistical difference between LAI and UAI when bacteria reduction was used to compare treatment efficacy. They showed that UAI, LAI and standard syringe irrigation protocols all achieved a median and mean CFU/mL reduction from approximately 10^8 to 10^6. These bacterial levels are probably not conducive with periapical health given that their post-treatment levels were higher than the pre-treatment bacteria levels (6.7×10^4 CFU/mL) of teeth with clinical and radiographic evidence of apical periodontitis (Byström & Sundqvist, 1981; 1985; Sjögren *et al.*, 1991; Peters *et al.*, 2002; Vianna *et al.*, 2006) . This study by Peters *et al.* (2011) shows that low bacteria levels are not consistently achieved when 6% NaOCl is either ultrasonically or laser activated for a period of 60 seconds. The present study shows viable bacteria number may be consistently reduced to low levels in teeth with complex root canal anatomy if both chemomechanical preparation and UAI or LAI regimes are used.

Neelakantan *et al.* (2014) found UAI, LAI and standard irrigation to be equally effective at reducing bacteria number when 3% NaOCl and 17% EDTA were used in a NaOCl-EDTA-NaOCl combination with a total activation time of 4 minutes and contact time of 6 minutes. Neelakantan *et al.* (2014) also used a mixed solution of 3% NaOCl and 18% etidronic acid. Interestingly, LAI was found to be statistically more effective at removing bacteria from the dentine tubules than UAI and syringe irrigation protocols. The increased efficacy of LAI may be related to the etidronic acids continuous chelating effect which may have allowed better penetration of NaOCl into the dentine tubules thereby enabling bacteria reduction. The present study used a similar irrigating protocol to the NaOCl-EDTA-NaOCl used by Neelakantan *et al.* (2014) and also showed that no difference was found between the treatment protocols.

4.9 Canal curvature and its impact on treatment efficacy

Teeth that were classified as severely curved (30≥ degrees) were significantly more difficult to disinfect than teeth that were either mildly or moderately curved. The delivery of the irrigant into the apical third via a needle is problematic (Tay *et al.*, 2010). Increasing

the taper and apical preparation size improves irrigant delivery to the apical area in both straight (Falk & Sedgley, 2005; Boutsioukis *et al.*, 2014) and curved canals (Nguy & Sedgley, 2006). Bacteria reduction is increased when the needle tip is placed within close proximity to the apex (Sedgley *et al.*, 2005b). The present study prepared the canals to 35/.06 using a NiTi file system that preserves the canal curvature (Gergi *et al.*, 2014) and enabled placement of the needle tip to within 1-2 mm of the working length in roots that were mildly to moderately curved. Irrigant flow to the apical area decreases as the tip of the needle moves away from the apex towards the coronal segments of the canal (Boutsioukis *et al.*, 2010b). It was noted in the present study that access to the apical third became restricted as the curvature of the canal increased. This most likely decreased the flow of irrigant in the apical third of the canal in severely curved canals and may be the reason why severely curved roots in this study contained more bacteria after the chemomechanical preparation than mildly or moderately curved roots.

In the present study the tip of the ultrasonic file was positioned coronal to the curvature where the file could be heard to be oscillating without significant canal wall impediment. Recent research has shown that ultrasonic activation of an irrigant does improve the flow of the irrigant beyond the tip of the file towards the apex in curved canals (Merino *et al.*, 2013). Improving the flow of irrigant into the apical area may improve the efficacy by increasing the availability and refreshment of chlorine ions while also improving the removal of the dentine debris (Malki *et al.*, 2012). It is not possible to determine from the present study if UAI improved the flow of NaOCl into the apical third of the canal where canal curvature is most extreme.

No study, other than the present, has investigated the efficacy of LAI in multi-rooted or teeth with curved canals. The number of roots with severely curved canals in the LAI group was low, therefore it was not possible to determine whether there is any statistical difference between severely curved and mildly or moderately curved root when the LAI protocol was used.

4.10 The efficacy of the treatment protocols as determined by qPCR

The total bacteria load (10^6 - 10^7) recovered from roots of the control teeth (No Tx) in the present study was higher than reported by *in vivo* studies using qPCR and universal

primers (Vianna *et al.*, 2006; Blome *et al.*, 2008; Saito *et al.*, 2009; Paiva *et al.*, 2013a; b; Rôças *et al.*, 2013; Neves *et al.*, 2014). The reasons for this are most likely due to the differences in *ex vivo* or *in vivo* infections, nutrient supply, sampling method and the significant anatomical and volumetric variations that may exist between the root canal systems of roots with single and multi-canal roots with complex anatomical features.

Quantitative PCR was used to measure the efficacy of various treatment protocols in the present study. All treatment protocols removed significant amounts of bacteria from the root canal system when compared to roots that received no treatment. This is in agreement with virtually all studies on the subject (Vianna *et al.*, 2006; Blome *et al.*, 2008; Saito *et al.*, 2009; Paiva *et al.*, 2013a; b; Rôças *et al.*, 2013; Neves *et al.*, 2014).

Similar numbers of bacteria were removed from the root canal system using mechanical preparation and either saline irrigation, UAI (4% NaOCl and 15% EDTAC) or LAI (4% NaOCl and 15% EDTAC). This was an unexpected finding and is difficult to explain, especially given that culturing based studies have shown that NaOCl is a more effective antibacterial solution than saline (Byström & Sundqvist, 1981). SEM analysis showed a thick smear layer lined most areas of the canal wall in teeth irrigated with saline however canals that were irrigated with NaOCl and EDTA showed only isolated areas where bacteria and smear layer were present. It is not known whether this finding is unique to the present study or not because no other study has used qPCR to investigate the effect of NaOCl in comparison to saline irrigation on bacteria DNA from the root canal system.

Chemomechanical preparation supplemented with UAI or LAI using 4% NaOCl and 15% EDTAC reduced the bacteria DNA load by approximately 1-1.5 log fold. This value is lower than reported in other studies that prepared the straight canals using traditional chemomechanical procedures and showed a 2-3 log fold reduction in bacteria DNA (Blome *et al.*, 2008; Saito *et al.*, 2009; Paiva *et al.*, 2013a; b; Rôças *et al.*, 2013; Neves *et al.*, 2014). This is the first study to use qPCR to calculate the total bacterial DNA that may remain within the lower molar mesial root canal system after treatment. It is currently not known how much bacteria DNA may accumulate in the lower molar mesial root isthmus region and the apical region of curved canals. It is also expected that crushing the roots would have greatly improved the detection of bacteria DNA residing deep within the root canal system and in dentine tubules. These factors may account for the higher remaining

bacteria number as determined by qPCR in this study.

4.11 Comparison between culturing and qPCR methods

Culturing results are clinically relevant because a colony-forming unit represents at least one viable cell that has the capacity to cause disease if it remains within the root canal system after treatment. In the present study, viable bacteria, as determined by culturing, were found in the majority of roots after treatment, therefore culturing methods were an effective means of distinguishing the efficacy of each treatment group.

Quantitative PCR is a far more sensitive method of detecting the presence of bacteria than culturing methods (Vianna et al., 2006). The clinical significance of qPCR that quantifies DNA levels are still being determined, especially given that it does not discriminate between dead or live bacteria (Brundin et al., 2010). Quantitative PCR is a useful quantitative method if a lot of the samples have negative culturing results. This is because, even if qPCR may not be able to distinguish between dead or alive bacteria, it is an indicator for bioburden levels that may remain after treatment. If one treatment group is more effective in removing bioburden than another, the treatment that reduces the most bioburden is the preferred option (Rôças et al., 2013).

4.12 Clinical considerations

Extrusion of cytotoxic NaOCl through the apical foramen is deleterious to the surrounding periapical tissues and a clinical concern. Laser activation of an irrigant with high power settings (1.25 W) has been shown to extrude irrigant beyond the apex (George & Walsh, 2008) when the laser tip is placed within 5 mm from the apex. This study however was an *in vitro* study where there were no periapical tissues to provided resistance to the flow of the irrigant. Arslan et al. (2014) used a sponge to replicate the periapical tissues and collect any extruded irrigant. By determining the weight of the sponges the authors found LAI (0.3 W and 0.9 W) to extrude similar amounts of irrigant as UAI and traditional syringe irrigation. These findings confirm a *in vivo* recent study of 181 patients in which radiopaque contrast medium was mixed with NaOCl and used as the irrigant. Before laser activation, the teeth were chemomechanically prepared using traditional

methods and the pulp chamber was filled with irrigant. During activation, the laser tip was hovered above the canal orifices. Radiographically there was no contrast medium observed in the periapical tissues after laser activation. The authors did however report that 6% of the patients reported feelings of discomfort and pain, and even bleeding, although no further symptoms were reported. Even though this number is relatively low, it is still concerning that the laser created enough positive pressure in the apical tissues to cause pain. Very little is known about what parameters should be used to improve patient safety while maintaining the effectiveness of the laser energised irrigation. Further research is required before LAI can be fully recommended for routine clinical use.

Financial costs are a major limitation to the widespread implementation of any new technology. Erbium lasers and single use laser tip are expensive and also relatively cumbersome to move and store within the confines of a normal dental surgery. Recently, Neelakantan et al. (2014) showed that the Ezlase diode laser (940 nm wavelength, 50-60 Hz, continuous wave mode, with a maximum output power of 7W and a 200 μm plain ended fibre) and 6% NaOCl was as effective at removing mature *E. faecalis* biofilm as LAI using an erbium laser. Diode lasers are considerably smaller (hand held or bench top) and less expensive (< $10,000 AUS) than the larger (floor mounted) erbium lasers (~$85,000) and may be a promising alternative. However more research is necessary because it is unknown whether the effectiveness of the diode laser is due to cavitation or heating of the NaOCl.

Laser activated irrigation may be a potentially promising adjunct to traditional chemomechanical procedures however this is yet to be shown. Although it is has been advocated, LAI is unlikely to replace mechanical preparation of the root canal of the tooth which not only improves the delivery of irrigation solutions but also creates a space that facilitates predictable obturation.

5 Conclusion

This study showed that there is no significant difference between the effectiveness of LAI and UAI when used as a supplementary protocol to traditional chemomechanical procedures. Given that ultrasonic units are widespread and relatively inexpensive and safe to use, UAI is far more likely to be incorporated into routine general dental practices than LAI.

6 Appendices

6.1 Allocation of tooth roots to treatment groups for each flow cell

Flow cell		A	B	C	D	Total A-D
No Treatment	Two foramen	2	2	-	-	4
(Purple)	Common foramen	-	1	2	-	3
	Total					**7**
Saline SI	Two foramen	3	3	2	2	10
(Yellow)	Common foramen	2	3	3	2	10
	Total					**20**
UAI	Two foramen	3	1	4	1	9
(Blue)	Common foramen	5	1	4	1	11
	Total					**20**
LAI 0.5 W	Two foramen	3	1	4	2	10
(Peach)	Common foramen	5	1	4	2	12
	Total					**22**
LAI 0.75 W	Two foramen	-	4	-	4	8
(Pale peach)	Common foramen	-	6	-	8	14
	Total					**22**
SEM	No Treatment	1	-	-	-	1
	Saline SI	-	1	-	-	1
	UAI	-	-	1	-	1
	LAI 0.5W	-	-	-	1	1
	LAI 0.75W	-	-	-	1	1
	Total					**5**
Total tooth roots in flow cells		24	24	24	24	**96**

Table 9. Allocation of tooth roots to treatment groups for each flow cell.

6.2 Schematic diagrams of tooth root positions for flow cells A-D

Flow cell A

1 (I) **SEM**	6 (I)	11 (II)	15 (II)	20 (I)
2 (II)	7 (II)	12 (I)	16 (I)	21 (II)
3 (I)	8 (II)		17 (II)	22 (II)
4 (II)	9 (I)	13 (I)	18 (I)	23 (II)
5 (II)	10 (I)	14 (I)	19 (I)	24 (I)

Flow cell B

1 (I) **SEM**	6 (II)	11 (I)	15 (II)	20 (II)
2 (II)	7 (II)	12 (I)	16 (I)	21 (I)
3 (I)	8 (II)		17 (I)	22 (I)
4 (II)	9 (I)	13 (II)	18 (I)	23 (I)
5 (I)	10 (I)	14 (II)	19 (II)	24 (II)

Flow cell C

1 (II) **SEM**	6 (I)	11 (I)	15 (I)	20 (II)
2 (I)	7 (II)	12 (I)	16 (I)	21 (I)
3 (II)	8 (II)		17 (II)	22 (II)
4 (I)	9 (II)	13 (I)	18 (II)	23 (II)
5 (I)	10 (I)	14 (I)	19 (I)	24 (II)

Flow cell D

1 (II) **SEM**	6 (I)	11 (I)	15 (I)	20 (II)
2 (II) **SEM**	7 (II)	12 (I)	16 (II)	21 (I)
3 (II)	8 (II)		17 (I)	22 (I)
4 (I)	9 (II)	13 (II)	18 (I)	23 (II)
5 (I)	10 (I)	14 (I)	19 (I)	24 (II)

Figure 20. Schematic diagrams of tooth root positions for flow cells A-D.
The number in each square indicates the tooth root position in the flow cell. The Roman numeral in brackets indicates the foramen type: (I) = common foramen; (II) = two foramen. Each square is colour coded and corresponds to the treatment regimen: Purple: Group 1 – No Tx; Yellow: Group 2 – Saline SI; Blue: Group 3 - UAI; Peach: Group 4 – LAI 0.5 W: Pale peach: Group 5 – LAI 0.75 W. Tooth roots allocated for SEM analysis are marked.

6.3 Amplification profile of the primer validation assay

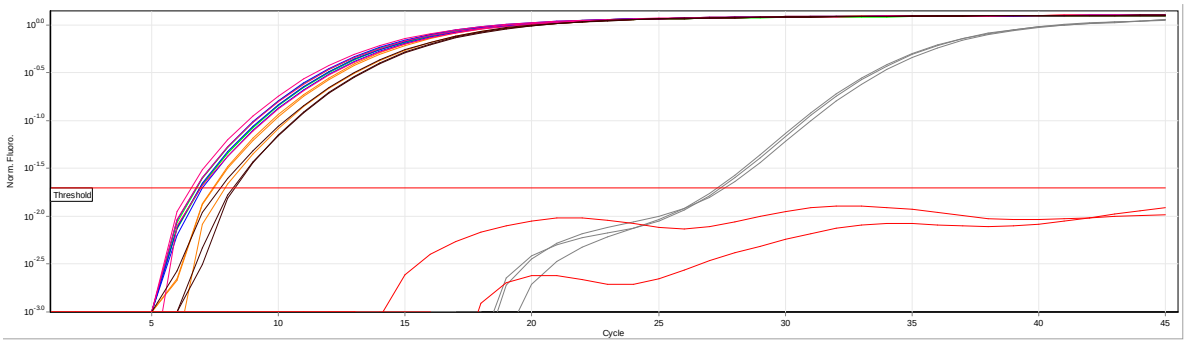

Figure 21. Amplification profile of the primer validation assay.
The multiple coloured (Table 10 gives the colour key) lines are the amplification profile of the 5 ng/µL stock solution DNA. The no template control (grey lines) Ct values are below the limit of detection as determined by the standard curve.

6.4 Primer validation assay data

Colour Key	Bacteria 5 ng/µl DNA stock solutions	Estimated theoretical 16S rRNA gene copy number	Ct	Average Ct value (S.D.)	Tm (ºC) value
	F. nucleatum	2.1 X 10^7	6.71	6.72 (± 0.21)	85.0
	F. nucleatum	2.1 X 10^7	6.94		85.2
	F. nucleatum	2.1 X 10^7	6.51		85.0
	Mixed species 10^0	1.55 X 10^7	6.73	6.84 (± 0.09)	86.0
	Mixed species 10^0	1.55 X 10^7	6.9		86.0
	Mixed species 10^0	1.55 X 10^7	6.89		86.0
	S. sanguinis	1.53 X 10^7	6.72	6.86 (± 0.15)	86.5
	S. sanguinis	1.53 X 10^7	7.01		86.5
	S. sanguinis	1.53 X 10^7	6.86		86.5
	E. faecalis	1.09 X 10^7	7.88	7.56 (± 0.28)	86.2
	E. faecalis	1.09 X 10^7	7.38		86.2
	E. faecalis	1.09 X 10^7	7.41		86.2
	Flow cell C	Unknown	7.7	8.05 (± 0.31)	86.2
	Flow cell C	Unknown	8.19		86.2
	Flow cell C	Unknown	8.26		86.2
	NTC		27.47	27.47 (± 0.14)	85.0
	NTC		27.33		85.5
	NTC		27.61		85.2
	dH_2O				
	dH_2O				

Table 10. Primer validation assay data.

6.5 Primer concentration optimisation

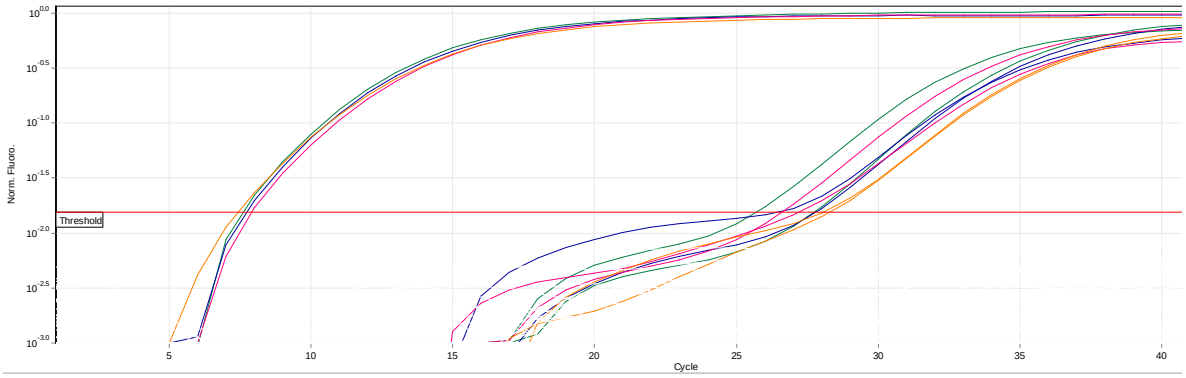

Figure 22. Primer concentration optimisation.

6.6 Primer concentration optimisation data

Bacteria 5 ng/µl DNA stock solutions	**Primer Concentration (pmole)**		**Ct value**	**Tm (ºC) value**
	Forward	**Reverse**		
Mixed species (10^0)	500	500	7.57	86.0
Mixed species (10^0)	500	250	7.71	85.5
Mixed species (10^0)	250	500	7.90	85.5
Mixed species (10^0)	250	250	7.41	85.5

Table 11. Primer concentration optimisation data.
Ct and Tm (ºC) value for the mixed species DNA stock solution using different forward and reverse primer concentrations.

6.7 Melting curve analysis for the primer validation assay

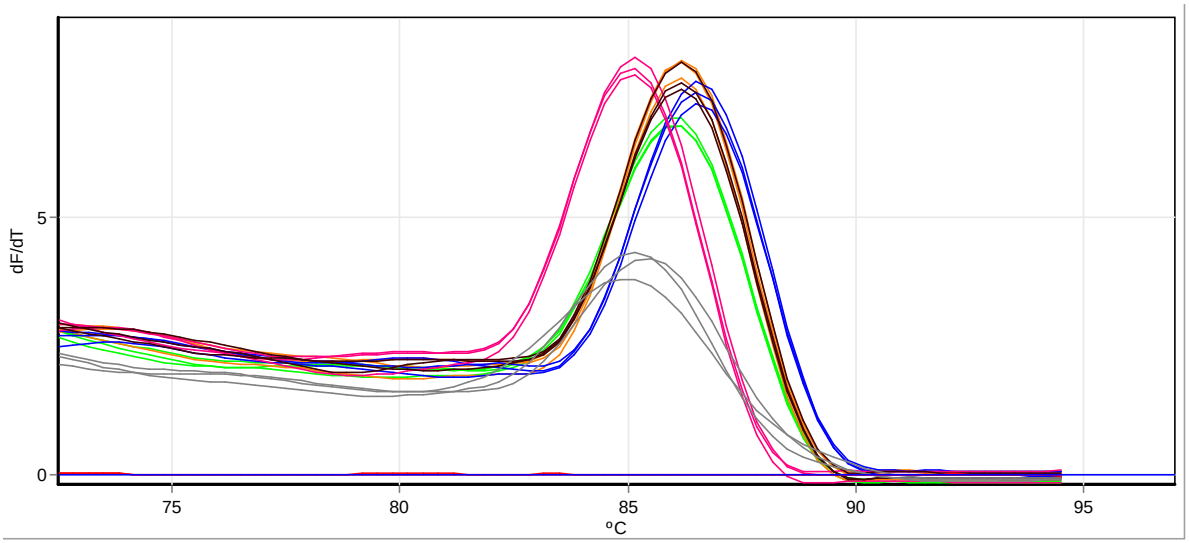

Figure 23. Melting curve analysis for the primer validation assay.
The x-axis is the melting temperature (ºC) of the dissociating amplicons and the y-axis is the fluorescence (-dF/dT). Table 10 gives the colour key.

6.8 Standard curve for mixed species qPCR

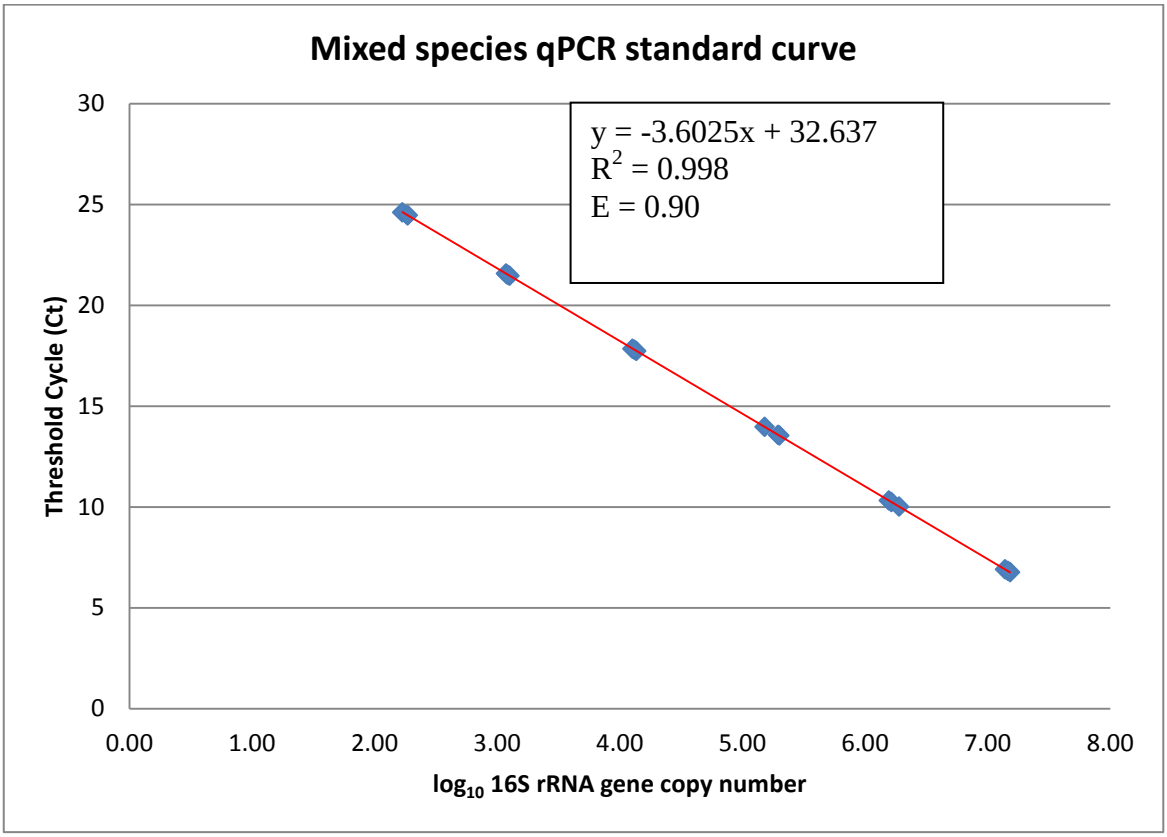

Figure 24. Standard curve for mixed species qPCR.

6.9 Data for the mixed species qPCR standard curve

Serial Dilution	Ct	Expected theoretical concentration (16S rRNA gene copies /reaction)	Calculated concentration (16S rRNA gene copies /reaction)	Log_{10} 16S rRNA gene copy number	Amplicon melting temperature (ºC) value
10^{0}	6.9	15500000	13906235	7.14	86.2
10^{0}	6.76	15500000	15263663	7.18	86.2
10^{0}	6.82	15500000	14647523	7.17	86.2
10^{-1}	10.32	1550000	1561992	6.19	86.2
10^{-1}	10.24	1550000	1645256	6.22	86.2
10^{-1}	10.02	1550000	1903751	6.28	86.2
10^{-2}	13.54	155000	200502	5.30	86.2
10^{-2}	13.97	155000	152277	5.18	86.2
10^{-2}	13.58	155000	195237	5.29	86.2
10^{-3}	17.85	15500	12720	4.10	86.2
10^{-3}	17.74	15500	13654	4.14	86.0
10^{-3}	17.8	15500	13136	4.12	86.2
10^{-4}	21.47	1500	1260	3.10	86.0
10^{-4}	21.57	1500	1179	3.07	86.0
10^{-4}	21.52	1500	1219	3.09	86.0
10^{-5}	24.46	150	186	2.27	86.0
10^{-5}	24.61	150	169	2.23	85.8
10^{-5}	24.46	150	186	2.27	86.0
10^{-6}	27.42	16	10	0.98	85.5
10^{-6}	28.64	16	4	0.61	86.2
10^{-6}	26.74	16	16	1.20	-
10^{-7}	27.41	1.55	10	0.99	85.8
10^{-7}	27.97	1.55	7	0.81	85.8
10^{-7}	27.88	1.55	7	0.84	-
NTC	28.34	0	16	1.19	86.0
NTC	27.11	0	34	1.53	85.7
NTC	27.57	0	25	1.41	85.5
dh20					
dh20					

Table 12. Data for the mixed species qPCR standard curve.
N/B Standards 10^{-6} and 10^{-7} were excluded from the standard curve construction

6.10 Amplification profile for the mixed species qPCR standard curve

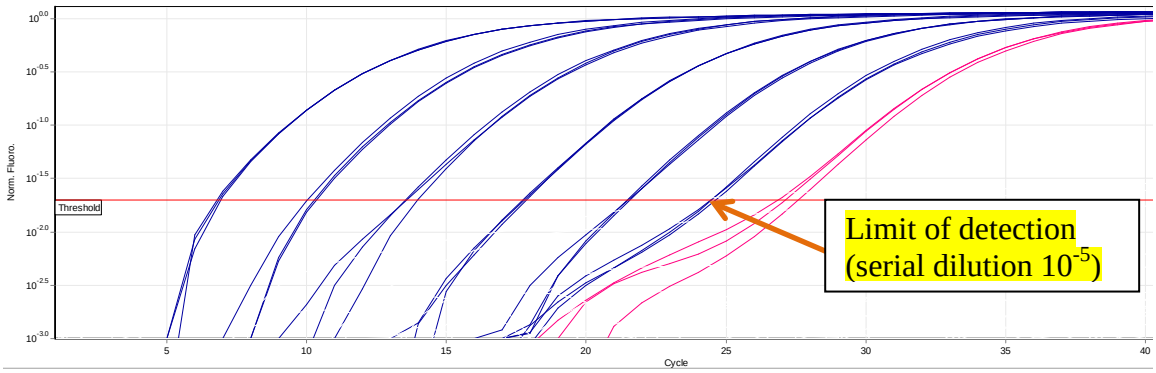

Figure 25. Amplification profile for the mixed species qPCR standard curve.
The blue lines are the amplification profile of the standard serial dilutions (10^{0} to 10^{-5}). The limit of detection (serial dilution 10^{-5}) was determined to be approximately 150 16S rRNA gene copies or 35 bacteria cells. The pink lines indicate no template controls that are well below the lower limit of detection.

6.11 Melting curve analysis for the mixed species standard curve

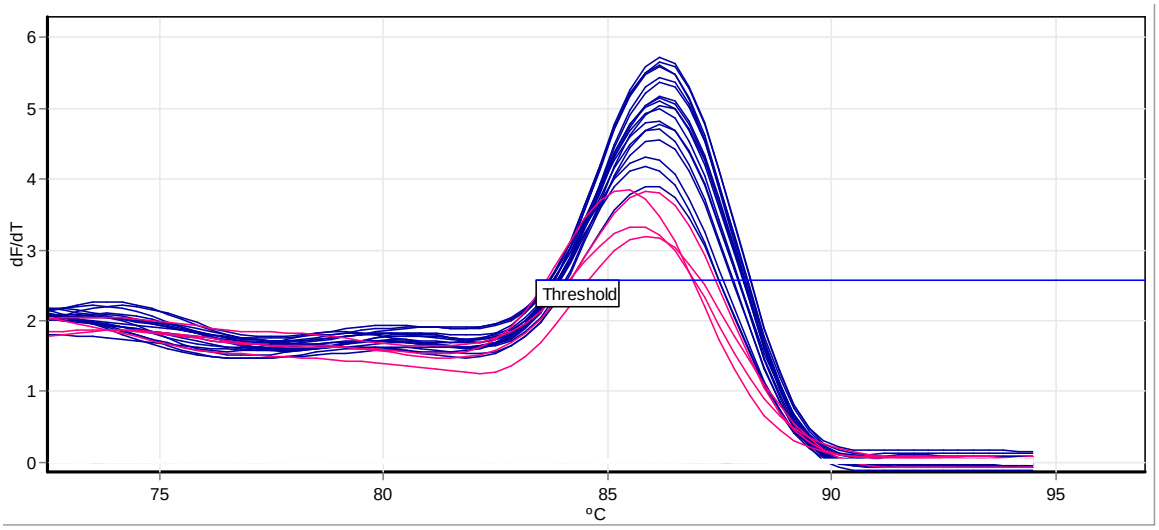

Figure 26. Melting curve analysis for the mixed species standard curve.
The x-axis is the melting temperature (ºC) of the dissociating amplicons and the y-axis is the fluorescence (-dF/dT). The blue lines are the standard curve serial dilutions (10^{0} to 10^{-5}) whereas the pink lines indicate no template controls.

6.12 Flow cell A

Flow cell	Position	Foramen type	Curvature group	Treatment group	CFU/mL	qPCR (cells/mL)
A	2	2	2	No Tx	Error	Error
A	15	2	1	No Tx	6.30E+04	2.06E+07
A	3	1	2	Saline SI	Error	Error
A	4	2	2	Saline SI	2.25E+03	8.89E+04
A	5	2	1	Saline SI	2.73E+03	8.26E+04
A	6	1	1	Saline SI	5.60E+04	2.29E+07
A	7	2	3	Saline SI	1.92E+04	8.39E+05
A	8	2	1	UAI	0.00E+00	1.45E+05
A	9	1	2	UAI	5.00E+01	8.14E+05
A	10	1	3	UAI	2.13E+03	7.12E+05
A	11	2	1	UAI	3.55E+02	3.57E+04
A	12	1	3	UAI	2.90E+02	1.93E+05
A	13	1	3	UAI	1.75E+03	9.14E+04
A	17	2	2	UAI	6.00E+01	3.36E+05
A	19	1	2	UAI	1.65E+02	3.80E+05
A	14	1	1	LAI 0.5 W	0.00E+00	1.65E+05
A	16	1	2	LAI 0.5 W	1.48E+03	1.26E+06
A	18	1	2	LAI 0.5 W	0.00E+00	1.27E+05
A	20	1	3	LAI 0.5 W	3.50E+01	1.43E+05
A	21	2	3	LAI 0.5 W	5.50E+01	2.74E+05
A	22	2	2	LAI 0.5 W	8.50E+01	2.79E+05
A	23	2	1	LAI 0.5 W	2.80E+02	2.05E+05
A	24	1	3	LAI 0.5 W	1.45E+02	1.50E+06
A	1	1	1	No Tx (SEM)	-	-

Table 13. Flow cell A data.

6.13 Flow cell B

Flow cell	Position	Foramen type	Curvature group	Treatment group	CFU/mL	qPCR (cells/mL)
B	2	2	1	No Tx	6.18E+03	3.77E+07
B	3	1	2	No Tx	4.52E+03	9.94E+06
B	4	2	2	No Tx	1.29E+04	7.15E+06
B	5	1	1	Saline SI	1.36E+03	6.46E+06
B	6	2	1	Saline SI	1.45E+04	7.96E+06
B	7	2	3	Saline SI	4.45E+02	5.43E+06
B	8	2	2	Saline SI	1.45E+02	1.08E+06
B	9	1	2	Saline SI	4.38E+03	5.28E+06
B	10	1	2	Saline SI	4.20E+02	2.29E+06
B	23	1	2	UAI	0.00E+00	7.02E+05
B	24	2	2	UAI	7.95E+02	1.44E+06
B	11	1	2	LAI 0.5 W	7.50E+01	4.59E+06
B	13	2	1	LAI 0.5 W	0.00E+00	1.90E+06
B	12	1	2	LAI 0.75 W	0.00E+00	1.32E+06
B	14	2	2	LAI 0.75 W	4.23E+03	9.32E+05
B	15	2	2	LAI 0.75 W	8.00E+02	6.50E+05
B	16	1	1	LAI 0.75 W	9.05E+02	8.44E+05
B	17	1	2	LAI 0.75 W	0.00E+00	1.34E+06
B	18	1	1	LAI 0.75 W	1.06E+03	9.35E+05
B	19	2	2	LAI 0.75 W	2.64E+03	2.59E+06
B	20	2	3	LAI 0.75 W	0.00E+00	5.01E+05
B	21	1	1	LAI 0.75 W	2.95E+02	4.92E+05
B	22	1	1	LAI 0.75 W	4.10E+02	1.52E+06
B	1	1	2	Saline SI (SEM)	-	-

Table 14. Flow cell B data.

6.14 Flow cell C

Flow cell	Position	Foramen type	Curvature group	Treatment group	CFU/mL	qPCR (cells/mL)
C	2	1	2	No Tx	Error	Error
C	14	1	2	No Tx	3.37E+05	1.82E+06
C	3	2	1	Saline SI	6.35E+02	4.07E+06
C	4	1	2	Saline SI	1.13E+03	2.81E+06
C	5	1	1	Saline SI	2.92E+04	3.05E+06
C	6	1	2	Saline SI	2.89E+04	3.50E+06
C	7	2	1	Saline SI	2.65E+02	6.41E+05
C	8	2	2	UAI	1.85E+02	3.19E+06
C	9	2	2	UAI	1.79E+03	1.71E+06
C	10	1	1	UAI	0.00E+00	1.19E+06
C	11	1	3	UAI	4.55E+02	7.58E+05
C	12	1	1	UAI	0.00E+00	3.77E+05
C	13	1	2	UAI	5.25E+02	1.49E+06
C	17	2	2	UAI	1.00E+01	5.83E+05
C	18	2	1	UAI	1.00E+01	1.10E+05
C	15	1	2	LAI 0.5 W	1.05E+02	2.35E+05
C	16	1	1	LAI 0.5 W	1.00E+02	6.68E+05
C	19	1	1	LAI 0.5 W	3.20E+02	5.18E+05
C	20	2	2	LAI 0.5 W	3.50E+01	5.76E+05
C	21	1	1	LAI 0.5 W	1.50E+01	2.03E+05
C	22	2	1	LAI 0.5 W	3.60E+02	1.62E+06
C	23	2	2	LAI 0.5 W	4.00E+01	1.66E+06
C	24	2	1	LAI 0.5 W	9.50E+01	1.00E+06
C	1	2	2	UAI (SEM)	-	-

Table 15. Flow cell C data.

6.15 Flow cell D

Flow cell	Position	Foramen type	Curvature group	Treatment group	CFU/mL	qPCR (cells/mL)
D	21	1	2	Saline SI	-	-
D	22	1	2	Saline SI	-	-
D	23	2	2	Saline SI	-	-
D	24	2	2	Saline SI	-	-
D	3	2	2	UAI	-	-
D	4	1	2	UAI	-	-
D	5	1	1	LAI 0.5 W	-	-
D	8	2	2	LAI 0.5 W	-	-
D	11	1	1	LAI 0.5 W	-	-
D	13	2	2	LAI 0.5 W	-	-
D	6	1	2	LAI 0.75 W	-	-
D	7	2	2	LAI 0.75 W	-	-
D	9	2	2	LAI 0.75 W	-	-
D	10	1	1	LAI 0.75 W	-	-
D	12	1	1	LAI 0.75W	-	-
D	14	1	2	LAI 0.75W	-	-
D	15	1	2	LAI 0.75W	-	-
D	16	2	2	LAI 0.75W	-	-
D	17	1	1	LAI 0.75W	-	-
D	18	1	2	LAI 0.75W	-	-
D	19	1	1	LAI 0.75W	-	-
D	20	2	2	LAI 0.75W	-	-
D	1	2	2	LAI 0.5 W (SEM)	-	-
D	2	2	2	LAI 0.75 W (SEM)	-	-

Table 16. Flow cell D data.
All results from flow cell D were discarded from quantitative analysis due to contamination however two roots were used for qualitative SEM analysis.

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