

**EVALUATION OF THE EFFECTIVENESS OF TWO IRRIGATION SYSTEMS IN  
REDUCING BACTERIAL LOAD IN ROOT CANALS IN VITRO BY QUANTITATIVE  
REAL-TIME POLYMERASE CHAIN REACTION**

by

Duo Zhang

D.M.D, Sichuan University, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES  
(Craniofacial Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2017

© Duo Zhang, 2017

## **Abstract**

**Introduction:** Irrigation is regarded as having a key role in one of the main goals of endodontic treatment, the killing and removing bacteria from the infected root canal. Different methods and techniques have been used to deliver irrigating solutions and to facilitate the effectiveness of irrigation. The purpose of this in vitro study was, by using quantitative real-time PCR to determine the effectiveness of two irrigation and cleaning systems in removing multispecies oral biofilms from root canals.

**Methods:** Fifteen extracted human molars were instrumented to size #15/.02 and then cleaned with the GentleWave System (GW; Sonendo Inc, Laguna Hills, CA). The teeth were autoclaved to provide the same sterile baseline. The molars were filled with mixed plaque suspended in brain-heart infusion broth (BHI; Becton Dickinson, Sparks, MD) and centrifuged to help the bacteria spread all over the root canal system. After two weeks of incubation, samples were divided randomly into two treatment groups and instrumented into size #15/.04 (GW) and #35/.04 (PiezoFlow group) under needle irrigation with sterile water. The teeth were then cleaned either with GentleWave System or ProUltra PiezoFlow Active Ultrasonic System (Dentsply Tulsa, Tulsa, OK) using 3% NaOCl, 8% EDTA and sterile water as irrigants. Root canals were sampled with paper points before and after instrumentation (S1, S2) and after GW or PF cleaning (S3). Quantitative real-time PCR was performed, the presence of microorganism in the samples was determined by using universal bacterial, a genus specific and species-specific primers. Statistical analysis was performed using the Mann-Whitney U test with the significance level set at  $P < 0.05$ .

**Result:** A highly significant bacterial reduction was recorded for both groups ( $P < 0.001$ ). GW group showed a significantly higher reduction than the PF group of total microbes ( $P < 0.01$ ), *Enterococcus faecalis* ( $P < 0.01$ ) and *Streptococcus* species ( $P < 0.05$ ). GW resulted predictably in high reduction of over 99% of the microbes in every canal, whereas in the PF group the reduction of microbes varied between 87% and 99.99%.

**Conclusion:** While both systems demonstrated effective reduction of intracanal bacteria, a high level of over 99% was more predictably achieved in the GW group.

## **Lay Summary**

Apical periodontitis is an infectious oral disease which starts when bacteria invade the root canal inside the tooth. Apical periodontitis can be identified in a radiograph as destruction of bone structures around the root tip of the affected tooth. Healing of the infection requires that bacteria inside the tooth are removed completely or reduced to a very low level. Eradication of bacteria, according to studies and clinical experience is difficult. In this study we examined the ability of a novel equipment specifically designed to clean infected root canals from bacteria for its efficacy and compared it to the most effective system so far. Sensitive, modern techniques were used to measure the effectiveness and compare two the systems. The results showed that the new system, GentleWave, produced cleaner canals with higher predictability than the other system.

## **Preface**

The study design was identified by Dr. Duo Zhang under the guidance of Dr. Markus Haapasalo and Dr. Ya Shen. Teeth collection, treatment, and sample taking was carried out by Markus Haapasalo and Prashanthi Vandurangi. DNA extraction and QPCR were performed by Dr. Duo Zhang. Data were collected and analyzed by Dr. Duo Zhang with the assistance of Dr. HsingChi von Bergmann. Thesis was prepared by Dr. Duo Zhang and revised by Dr. Markus Haapasalo and Dr. Ya Shen. Ethics approval was acquired from the University of British Columbia Office of Research Services, Clinical Research Ethics Board (certificate number H15-02793).

# Table of Contents

<b>Abstract.....</b>	<b>ii</b>
<b>Lay Summary .....</b>	<b>iv</b>
<b>Preface.....</b>	<b>v</b>
<b>Table of Contents .....</b>	<b>vi</b>
<b>List of Tables .....</b>	<b>ix</b>
<b>List of Figures.....</b>	<b>x</b>
<b>List of Abbreviations .....</b>	<b>xi</b>
<b>Acknowledgements .....</b>	<b>xiii</b>
<b>Dedication .....</b>	<b>xiv</b>
<b>Chapter 1: Literature Review.....</b>	<b>1</b>
1.1    Introduction.....	1
1.2    Biofilm .....	1
1.3    Apical Periodontitis .....	6
1.3.1    Location of the Microbes in the Root Canal System .....	7
1.3.2    Distribution of the Microbes in Various Parts of the Root Canal.....	9
1.4    Root Canal Treatment.....	10
1.5    Irrigation .....	11
1.5.1    Irrigating Solutions .....	12
1.5.2    Irrigation Methods .....	13
1.5.2.1    Conventional Syringe Needle Irrigation (CSNI) .....	13
1.5.2.2    Apical Negative Pressure System (ANP) .....	13

1.5.2.3	Passive Ultrasonic Irrigation System (PUI).....	14
1.5.2.4	Continuous Ultrasonic Irrigation (CUI).....	15
1.5.2.5	Laser Activated Irrigation (LAI) and Photon-Induced Photoacoustic Streaming (PIPS) .....	15
1.5.2.6	GentleWave System.....	16
1.5.3	Fluid Hydrodynamics.....	17
1.6	Methods of Sampling Bacteria from Root Canals .....	18
1.7	Quantification of the Biofilm Microbes.....	19
1.7.1	Culture Based Methods.....	19
1.7.2	qPCR.....	19
1.8	Aim of the Study.....	20
1.9	Hypothesis.....	21
<b>Chapter 2: Materials and Methods .....</b>		<b>22</b>
2.1	Tooth Collection and Preparation .....	22
2.2	Inoculation .....	22
2.3	Sampling of the Bacteria from the Canals, First Sample.....	23
2.4	GentleWave Group, Second and Third Sample .....	23
2.5	Ultrasonic System Group, Second and Third Sample .....	24
2.6	DNA Extraction and Quantitative Real-time PCR Analysis .....	24
2.7	Statistical Analysis.....	28
<b>Chapter 3: Results.....</b>		<b>29</b>
3.1	qPCR Co-efficiency Test and Specificity .....	29
3.2	qPCR Results of Bacterial Quantification .....	31

<b>Chapter 4: Discussion .....</b>	<b>36</b>
<b>Chapter 5: Limitations of the Study.....</b>	<b>41</b>
<b>Chapter 6: Conclusion.....</b>	<b>42</b>
<b>Chapter 7: Future Directions.....</b>	<b>43</b>
<b>References .....</b>	<b>44</b>
<b>Appendices.....</b>	<b>67</b>



## List of Tables

Table 2.1	Primers Used in Real-time PCR for Bacterial Quantification.....	25
Table 3.1	Level of Total Bacteria in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave/PF Ultrasonic System (S3) .....	31
Table 3.2	Level of <i>Enterococcus faecalis</i> in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave/PF Ultrasonic System (S3) .....	32
Table 3.3	Level of Streptococcus species in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave/PF Ultrasonic System (S3) .....	33

## List of Figures

Figure 3.1 Standard Curve of qPCR Co-efficiency Test.....	28
Figure 3.2 Melt Curve of qPCR Assay - qPCR Specificity.....	29

## List of Abbreviations

ANP.....	apical negative pressure system
ATCC.....	American Type Culture Collection
BHI.....	brain-heart infusion
CFD.....	Computational fluid dynamics
CFU.....	counting of colony-forming
CHX.....	chlorhexidine
CSNI.....	conventional syringe needle irrigation
CUI.....	continuous ultrasonic irrigation
DNA .....	deoxyribonucleic acid
EDTA .....	ethylenediaminetetraacetic acid
<i>E. faecalis</i> .....	<i>Enterococcus faecalis</i>
EPS.....	extracellular polymeric substance
LAI.....	laser activated irrigation
Min.....	minute
NaOCl .....	sodium hypochlorite
PBS.....	phosphate buffered saline
PIPS.....	photon-induced photoacoustic streaming
PMA.....	propidium monoazide
PTED.....	post-treatment endodontic disease
PUI.....	passive ultrasonic irrigation system
QPCR.....	quantitative polymerase chain reaction
Sec.....	second

UAI.....ultrasonically activated irrigation

WL.....working length

## **Acknowledgements**

Firstly, I owe my sincerest thankfulness to my supervisor Professor Markus Haapasalo, who is always willing to share his wisdom and expertise to me. His richness of endodontics knowledge, passionate enthusiasm in research and sense of humor motivate me to study and research more in endodontics. I am also grateful to his guidance during the whole project and providing me creative and valuable ideas on every step of my project. What I have achieved would not be possible without his patient guidance.

I also would like to express my sincere gratefulness to Dr. Ya Shen, who is my co-supervisor. Her kind support and professional guidance encourage me to overcome every difficulty in my project.

I am thankful to my committee members Dr. Lari Hakkinen and Dr. Clive R. Roberts for their valuable input to my study design and troubleshooting.

## **Dedication**

To my parents, thank you for your unwavering support and encouragement in my education and life. I can't achieve this without you.

To my wife, your accompanying and love are always my motivation to pursue our dream.

To Dr. Bo Hu, thank you for your kind care and support on both my education and future career.

Thank you for sharing your knowledge and experience.

# **Chapter 1: Literature Review**

## **1.1 Introduction**

Various factors such as chemical and physical irritants can cause irritation and even necrosis of the pulp, but bacteria and their products entering the pulp are the most common cause for endodontic diseases such as pulpitis and apical periodontitis (Bergenholtz 1974; Bergenholtz 1990). One of the primary goals of the endodontic treatment is to eradicate the microbes in root canal systems (Sjogren et al. 1997; Chugal et al. 2001; Jhajharia et al. 2015). Nowadays, there is still no recognized treatment strategy that can predictably eliminate all bacteria from infected root canals. The successful treatment of endodontic diseases depends on killing and removing as many microbes as possible from root canal system and preventing reinfection. Oral infections, root canal infections included, are biofilm infections. Therefore, successful control and prevention of oral infections depends on success in preventing and controlling oral biofilms.

## **1.2 Biofilm**

Traditionally, much of the research in endodontic disinfection has been done with pure cultures of planktonic bacteria, in test tube conditions (Gomes et al. 2001). Many of the available disinfecting agents are quite effective against planktonic microbes and killing may results in just a few seconds, even with low concentration of the disinfecting agent. These results were in stark contrast with the clinical reality, where it seemed particularly difficult to eradicate the microbes from the necrotic, infected root canal (Nair et al. 1990; Nair et al. 1999). The realization that endodontic microbes are organized as biofilms around the root canal system, has opened a new approach to the understanding of endodontic microbiology and disinfection in clinical

endodontics. Biofilm is a complex bacterial community in which cells attach to each other and are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) (Costerton et al. 1994; Costerton et al. 1999; Socransky and Haffajee 2002; Stoodley et al. 2002). EPS is a matrix material consisting of nucleic acid, polysaccharides, proteins and salt. Some of the bacteria have the ability to rapidly build a self-aggregating collection of a large number of bacterial cells embedded in EPS which helps them escape the host defense system (Nair et al. 2008). Because of the three-dimensional structure, the residing bacteria in a biofilm community have a metabolic cooperation between resident cells of different species. Thus, the bacteria in a biofilm have high resistance against the host's immune system and disinfecting agents (Gilbert et al. 1997; Costerton et al. 1999; Chavez de Paz et al. 2007). In the oral cavity, there are three basic steps involving biofilm formation: Pellicle formation, bacterial colonization and biofilm maturation (Gilbert et al. 1993; Jenkinson and Lappin-Scott 2001; Katsikogianni and Missirlis 2004). Oral biofilms are involved in the development of many oral diseases such as caries and periodontal diseases.

Bacterial cells, a fluid medium, and a solid surface are the three components involved in biofilm formation. The process of bacterial biofilm formation begins when a cell attaches to a surface coated with an organic conditioning polymeric matrix or “conditioning film” (Grenier and Mayrand 1986). In the initial phase, the contact between the cell and conditioning film is initially weak. Then the sticky exopolymers secreted by the cells help the bacterial cells to adhere to the surface irreversibly (Handley et al. 1985). In the next stage, the development of a biofilm occurs as a result of adherent cells replicating and by additional cells adhering to the biofilm (Jenkinson



and Lappin-Scott 2001). During the biofilm development and maturation process, some cells will eventually detach from the biofilm.

In endodontic infection, microorganisms penetrate into the pulpal space and spread further apically along the root canal. Biofilm formation in root canals is initiated after the invasion of the pulp chamber by oral organisms and pulp tissue breakdown (Mohammadi et al. 2013).

Nutritional and ecological status of the root canal has an impact on the progression of the infection. Development of an anaerobic environment and depletion of nutrition create a tough ecological niche for the surviving microorganisms. During root canal treatment, the anatomical and geometrical complexities in the root canal system shelter the biofilms from the mechanical cleaning procedures. A recent study indicated that the dead bacteria can provide the surviving bacteria a large amount of microbial biomass as a nutritional source for growth (Herrero et al. 2017). The presence of dead bacteria may have resulted in an outgrowth of several pathogenic species in complex multi-species biofilms. Additionally, upon contact with dead oral bacteria, virulence genes of *P. intermedia* and *P. gingivalis* were up-regulated. This resulted in a more pronounced epithelial cytotoxicity (Herrero et al. 2017).

### ***In vitro/ex vivo* models on extracted teeth**

Optimally, the most natural environment to do research on the effectiveness of disinfecting agents in endodontics would be natural teeth “in vivo” with apical periodontitis. However, because of numerous reasons, both ethical, practical and cost related, this is rarely possible. In addition, the wide variation between different teeth and different infections adds to the need of large patient material due to the confounding factors. The use of extracted teeth or prepared blocks of root

dentin *in vitro* and *ex vivo* has been a widely used approach in studies of the efficacy of endodontic disinfectants is an effort to better simulate the *in vivo* conditions and the *in vivo* reality of the root canal. Typically, just a single species, *E. faecalis*, or polymicrobial plaque collected from an infected root canal infection or from interdental space is incubated in the root canal (or partial root canal) for 1 – 30 days (Dunavant et al. 2006; Kishen et al. 2006; Shen et al. 2009; Williamson et al. 2009; de Paz et al. 2010; Shen et al. 2011; Stojicic et al. 2013). After the biofilm growth, different treatment protocols are performed, with an emphasis on various irrigation parameters, and microbiological samples e.g. for culturing are collected (Dunavant et al. 2006; Kishen et al. 2006; Williamson et al. 2009; Liu et al. 2010). Although these studies have provided much useful information, the dentin block model and other *ex vivo* models also have weaknesses and shortcomings. In many of these studies, the magnitude of biofilm growth and presence was not confirmed with any microscopic method. There are also great variations regarding the time of incubation of bacteria and how often nutrients were refreshed (Siqueira et al. 1997; Kishen et al. 2006; Bhuva et al. 2010; Liu et al. 2010). When the incubation is started, the microbes are mostly in a planktonic state, even exponential growth phase is possible. Such bacteria are extremely sensitive to disinfecting agents when compared to bacteria in established biofilms. Portenier et al. (2005) reported that bacteria in the starvation phase were 1,000 times more resistant to disinfecting agents than bacteria during the exponential or even stationary growth phase. In addition to the difference between planktonic bacteria in different growth phase, microbes in biofilms of different age and maturation level can demonstrate considerable differences in their susceptibility to antimicrobial agents (Shen et al. 2011; Stojicic et al. 2013).

## **Mono- and multi-species biofilms**

Research of *in vivo*, natural biofilms in their own environment is difficult. Therefore, efforts have been made to develop *in vitro* biofilm models which could closely mimic the key features of biofilms in the necrotic root canal of a tooth with apical periodontitis, e.g. In oral infections, the biofilms are almost always multispecies biofilms. In persistent endodontic infections, cultural studies have indicated that in some cases a monospecies *E. faecalis* infection may be involved (Peciulienė et al. 2000). Over the past years, endodontic research on biofilms has focused on both single-species (Du et al. 2013) and multi-species biofilm models (Shen et al. 2009; Arias-Moliz et al. 2010). In one study, the ability of four root canal bacteria to establish a multi-species biofilm community was studied with special focus on various structural and ecological aspects of the biofilm (de Paz 2012). The species included were *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus gordonii*, and *E. faecalis*, all originally isolated from infected root canals. The four species were able to develop stable *in vitro* biofilms with a relatively low proportion of dead cells. However, the overall time for biofilm growth examined was relatively short in this and many other experiments.

Several new biofilm models have been reported by a group from the University of British Columbia in recent years (Shen et al. 2009; Shen et al. 2010; Ma et al. 2011; Shen et al. 2011). These *in vitro* models include mono- and multispecies biofilms grown on collagen coated hydroxyapatite discs, dentin discs and in microscopic dentin canals (Shen et al. 2009; Shen et al. 2010; Ma et al. 2011; Shen et al. 2011). *E. faecalis* has been the most commonly used species in the monospecies biofilms, whereas the multispecies biofilms have been based on supra- and subgingival plaque, thus containing tens of different species, both facultative and anaerobic

bacteria. Anaerobic growth conditions and limited availability of nutrients (added only once per week) has been chosen as a strategy to invite the biofilm microbes to depend more on interbacterial interactions than continuous supply of nutrients from an external source. As a result, spirochetes are regularly detected in these biofilms even after extended periods of incubation for several weeks, something which has not been described earlier in literature for in vitro biofilms (Shen et al. 2009). In dentin canal biofilm model by the same group, centrifugation has been used as a method to force large numbers of bacteria into the dentinal tubules (Ma et al. 2011). Pure cultures of *E. faecalis* and mixed plaque have both been used in the dentin canal model with centrifugation. The method has made it possible to produce high numbers of parallel specimens with similar type of biofilm inside dentin, for the study of the effect of endodontic disinfecting agents in deeper layer of dentin (Wang et al. 2012; Du et al. 2014; Wang et al. 2014; Du et al. 2015; Yang et al. 2016).

### **1.3 Apical Periodontitis**

Apical periodontitis is an inflammatory lesion around the apex of a tooth root caused by biofilm-related infection in the necrotic root canal (Moller et al. 1981; Lee et al. 2016). The bacteria in teeth with apical periodontitis are mainly obligate anaerobic bacteria, which often constitute as much as 90% of the microbial flora (Wittgow and Sabiston 1975). The microbiota is a polymicrobial mix with approximately equal proportions of Gram-positive and Gram-negative species, dominated by obligately anaerobic bacteria (Figdor and Sundqvist 2007). Within the numerous species, *Enterococcus faecalis* and *Streptococcus* species are among those often isolated from infected root canals of human teeth (Marton and Kiss 2000; Wang et al. 2015; Tatikonda et al. 2017). *Enterococcus faecalis* is the most frequent species present in post-treatment disease and may play a significant role in persistent periapical infections following root canal treatment

(Sundqvist et al. 1998; Peciuliene et al. 2001; Pinheiro et al. 2003; Rocas et al. 2004; Siqueira and Rocas 2004; Skucaite et al. 2010). *Streptococcus mutans* is considered to be the microbial species most strongly associated with enamel caries and initiation of tooth surface biofilm formation (Nicolas and Lavoie 2011).

It has been previously estimated that about 500 species of bacteria inhabit the human oral cavity (Wilson et al. 1997). However, currently more than 1,000 bacterial species belonging to 13 phylotypes have already been identified in the oral microbiota and oral cavity (Wade 2013). Many of these species can invade the root canal system and thus are potential endodontic pathogens. Culture-based approaches have been widely used to identify microbes in infected root canals. The average number of isolated species per canal was reported from 2.4-6.8 (Aderhold et al. 1981; Brook et al. 1991; Sundqvist 1992; Brauner and Conrads 1995). Studies using molecular biological methods have shown that more than 200 different microbial species can be found in infected root canals, more than 20 species per canal can be detected (Rolph et al. 2001; Vianna et al. 2005). Over 900 bacterial species have been found within the root canal system by using barcoded multiplex pyrosequencing, a high-throughput multiplexed 16S rRNA gene barcoded pyrosequencing approach (Santos et al. 2011). Of these species, 486 were found in acute endodontic infections, 265 in chronic infections, and 165 in both cases (Santos et al. 2011).

### **1.3.1 Location of the Microbes in the Root Canal System**

The root dentin is hard tissue with countless ca. 2 µm wide dentin canals traveling from the pulp to the root surface cement. Several studies have shown that bacteria can and will penetrate the

dentin canals under favorable conditions Bacterial presence in dentinal tubules has been demonstrated in most teeth with necrotic canal and apical periodontitis.

Ørstavik & Haapasalo (1990) investigated the effect of endodontic antibacterial agents in dentin blocks prepared from bovine dentin. The authors culture bacteria in the root canal from where some bacteria penetrated into dentin canals. *E. faecalis* penetrated into the dentin canals more effectively than some other species. Other studies have reported bacterial penetration of 200 µm or more into dentinal tubules (Haapasalo and Orstavik 1987; Love and Jenkinson 2002) Lateral canals, which are small ramifications from the main root canal may also harbor bacteria/bacterial biofilm causing lateral lesions. Histological studies have shown that lateral canals are difficult or impossible to completely clean by presently available methods (Ricucci and Siqueira 2010). Therefore, bacteria in lateral canals can cause post-treatment endodontic disease (PTED).

The presence of a biofilm with complex structure has been reported occupying the isthmus of a tooth, which had been treated 10 years earlier and then re-treated two years from the original treatment. The persisting infection eventually lead to extraction of the tooth (Carr et al. 2009). The study emphasizes the importance of obtaining as complete cleanliness of the whole root canal system as possible.

In addition to established biofilms, instrumented root canals contain smear layer unless chemically removed after the instrumentation. Smear layers in teeth with apical lesion are likely to contain microorganisms and their antigens. Therefore, smear layer and the infective material embedded

into it should be removed during the irrigation (McComb and Smith 1975; Mader et al. 1984; Czonstkowsky et al. 1990; Sen et al. 1995).

### **1.3.2 Distribution of the Microbes in Various Parts of the Root Canal**

Root canal microbiota is extremely complex, close to 1000 different species have been reported in different studies (Siqueira and Rocas 2009; Santos et al. 2011; Wade 2013). While all of these bacteria and other microorganisms have found their ecological niche in the necrotic root canal, there are also great differences between the ecological requirements between different species. Availability of oxygen and type of nutrients are among the main factors affecting the growth of microorganisms in the root canal. Interbacterial relationships also play a big role in the ability of specific species to establish themselves in the various parts of the root canal, as many species are dependent on metabolic end products produced by some other species.

Özok et al. (2012) examined bacterial ecology of different parts of the necrotic root canal and found that the apical root canal favors selection of a more anaerobe microbiota than the coronal canal. Often the coronal canal is richer in nutrients from the oral cavity. As a result, fast growing and carbohydrate utilizing species take over the coronal canal flora (Kassen et al. 2000, Pham et al. 2009). In the apical canal limitation of nutrients may prevent overgrowth by a single or just a few species, and the survival of many species is in fact dependent on survival of other species as well, which seems to favor diverse anaerobic microbiota (Kreft 2004).

## **1.4 Root Canal Treatment**

Root canal treatment, after diagnosis and treatment decision, consists of mechanical instrumentation, irrigation, intracanal medicaments between appointments, root canal filling, and coronal restoration (Eckerbom et al. 1989; Siren et al. 1997; Sjogren et al. 1997; Haapasalo and Endal 2003). A variety of different factors will affect the prognosis of the treatment. These include the initial size of the periapical lesion, complexity of the root canal anatomy, type and location of the flora, and different types of mechanical and/or biological complications during the treatment (Nair et al. 1990). Sjogren et al. (1997) reported that complete elimination (as shown by negative culture result) of bacteria prior to root filling was essential to high success rate of the root canal treatment. Complete periapical healing was detected in 94% of cases with negative culture before root filling whereas in cases with positive culture (growth of bacteria) before the filling the success rate was only 68%.

Mechanical instrumentation and irrigation both play key roles in the eradication of microbes. Instrumentation removes necrotic tissue and microorganisms from the root canal, and irrigation with chemical agents optimally completes the removal of tissue and killing of microorganisms. Due to the anatomic irregularities of the root canal including isthmuses, fins and apical deltas, many areas in the root canal system are still untouched during the instrumentation (Wu and Wesselink 2001; Ricucci and Bergenholtz 2003). Therefore, irrigation has a great responsibility to reach those areas of the root canal wall that are beyond the reach of instrumentation (Haapasalo et al. 2014).



The procedures of conservative endodontics, mechanical, chemical and biological, only affect tissue and microbes which are within the confines of the root canal system. In some cases, bacteria have been shown to reside outside the root canal, on the root surface or as independent colonies in the periapical lesion as biofilm islands (Nair et al. 1999). In these situations, conservative root canal treatment cannot bring healing of the lesion without completing the treatment with an endodontic microsurgical procedure, e.g. apicoectomy, where ca. 3 mm of the root tip is cut off and a retrograde cavity of a minimum of 3 mm is filled with a retrograde filling material (Frenkel and Stellmach 1973; Kim and Kratchman 2006; Tsesis et al. 2006).

## **1.5 Irrigation**

The goal of irrigation is not only to dissolve pulp tissue and to remove bacteria from the root canal but also to remove the smear layer and dentin debris created during instrumentation (Baugh and Wallace 2005; Haapasalo et al. 2005). Studies have reported that the efficacy of irrigation can determine the residual number of bacteria in root canal system and have a great influence on the short and long term prognosis (Haapasalo et al. 2014; Plotino et al. 2016). A successful treatment outcome is contributed by a combination of optimal irrigants with chemical agents and an effective irrigation method. A large number of studies have focused on development of new irrigant solutions (Kovac and Kovac 2011; Singla et al. 2011; Mohammadi 2015; Goncalves et al. 2016). However, over several decades, Sodium hypochlorite (NaOCl) and EDTA are still the most important and popular irrigants in root canal treatment (Siqueira et al. 2000; Dunavant et al. 2006; Haapasalo et al. 2014). On the other hand, numerous innovative irrigation techniques have been developed.

### 1.5.1 Irrigating Solutions

NaOCl is the most important irrigants in root canal treatment (Siqueira et al. 2000; Dunavant et al. 2006). It effectively kills microbes and dissolves organic matter in the root canals (Cobankara et al. 2010; Paque et al. 2012). NaOCl can ionize into sodium ( $\text{Na}^+$ ) and  $\text{ClO}^-$  in water.  $\text{ClO}^-$  and hypochlorous acid ( $\text{HClO}$ ) both have strong antimicrobial effect,  $\text{HClO}$  being stronger than  $\text{ClO}^-$ . (Gomes et al. 2001; Zehnder 2006). The tissue dissolving effect of NaOCl is dependent on concentration, volume and contact time. In addition, increasing the temperature, flow, and surface tension of NaOCl improve its effectiveness (Stojicic et al. 2010). NaOCl is used in concentrations between 0.5 -6 %, recently even 8% solution has been adopted by many endodontic specialists.

Ethylenediaminetetraacetic acid (EDTA) is another important irrigant, used to finalize the removal of the smear layer. EDTA is used in concentration of 17%, 15% or 8%. It only affects the inorganic part of dentine and smear layer, therefore it is used as final rinse after NaOCl (Goldman et al. 1982; Yamada et al. 1983). If mixed or used alternatively, EDTA weakens the effect of NaOCl.

Chlorhexidine (CHX) is used by many dentists as a final solution after EDTA particularly in cases of persistent root canal infections (Russell and Day 1993). Since CHX has no effect of dissolving organic or inorganic tissue, it cannot replace NaOCl and be used as the only irrigant. Its antibacterial effectiveness is weaker than that of 5% NaOCl, close to 2% NaOCl (Ma et al. 2011; Wang et al. 2012).

## **1.5.2 Irrigation Methods**

### **1.5.2.1 Conventional Syringe Needle Irrigation (CSNI)**

CSNI is the classical method of irrigation in root canal treatment. It can be effective in cleaning the main root canal when used properly (Kahn et al. 1995; van der Sluis et al. 2006). One recent study reported that a flow rate of 4 mL/min can achieve the optimal effect of irrigant exchange and yet cause a relative low apical pressure, which is important in reducing the risk of extrusion (Park et al. 2013). The use of side-vented needles can provide safer irrigation than open-ended needles, which may cause higher apical pressure at the foramen (Shen et al. 2009). Depending on canal anatomy and size, it is often difficult for the needle to reach the apical canal. Therefore, apical vapor lock can form and further prevent the solutions from reaching to the apical area of the root canal (Boutsioukis et al. 2014). Different modifications such as side vented or flexible needles have been introduced to improve the effectiveness and safety of syringe-needle irrigation. CSNI is not as effective as some other methods using additional energy in cleaning the complex root canal system (Siu and Baumgartner 2010).

### **1.5.2.2 Apical Negative Pressure System (ANP)**

ANP has been introduced to simultaneously deliver the irrigant to and suction from the canal in order to obtain a good and safe flow of irrigant into the entire root canal system. EndoVac (KerrEndo, Orange County, CA, USA) is a commercial realization of the ANP principle. It delivers a constant flow of irrigant from a Master Delivery Tip, and at the same time remove debris and irrigant solution at the apical third by MacroCannula or MicroCannula using suction. Negative pressure irrigation contributes to improved safety as compared to positive pressure, and it has been proven to be significantly safer than CSNI (Malentacca et al. 2012). Also, when compared to

ultrasonic irrigation methods, teeth treated by ANP have shown less debris extrusion (Tambe et al. 2013). As for efficacy, some early studies have shown that the ANP can effectively clean the apical part of the root since it has been shown to allow the irrigants to safely reach the most apical canal, help overcome apical vapor lock, and prevent extrusion of irrigant and debris into the periapical tissue (Siu and Baumgartner 2010; Tambe et al. 2013). However, some recent studies reported ANP was only slightly better than CSNI in debridement efficacy, smear layer removal, and dentinal tubule penetration (Malentacca et al. 2017; Turkel et al. 2017).

### **1.5.2.3 Passive Ultrasonic Irrigation System (PUI)**

PUI is utilized after instrumentation and without the intent to enlarge, instrument, or impact the walls of the root canal. Unfortunately, however, even though the intent is not to make contact the root canal walls, this will occur due to the oscillating motion of the ultrasonic instrument tip. Because of this, the phrase ultrasonically activated irrigation (UAI) was recently suggested instead of passive ultrasonic instrumentation (Boutsioukis et al. 2013). The effectiveness of PUI/UAI to remove tissue and debris has been studied. PUI was reported to remove debris significantly better than CSNI (Tanomaru-Filho et al. 2015; Topcuoglu et al. 2015). De Greporio et al. (2010) compared the penetration of NaOCl into the simulated working length and lateral canals and found that PUI enhanced the NaOCl penetration into the lateral canals more than ANP. A large number of studies have reported a significant reduction in the number of bacteria following the use of PUI when compared with CSNI (Huque et al. 1998; Sjogren et al. 1998; Spoleti et al. 2003; Townsend and Maki 2009; Cachovan et al. 2013). Culturing and counting the colony forming units (CFU) was used as the method of quantification.

#### **1.5.2.4 Continuous Ultrasonic Irrigation (CUI)**

Although PUI was reported to improve the cleanliness of root canal system, the lack of irrigant replenishment is an issue. CUI was developed to resolve this issue. In CUI, the needle simultaneously activates and replenishes the irrigant in the canals. The high-power stream generated has a promising cleaning effect on the rough surfaces and otherwise difficult to reach areas within the root canal system. Castelo-Baz et al. (Castelo-Baz et al. 2016) reported that CUI with the Piezoflow™ system (Dentsply Tulsa, Tulsa, OK, USA) was more effective than PUI/UAI in getting irrigant into lateral canals. The authors found that using CUI as the final rinse can significantly increase the penetration of irrigant into the simulated lateral canals and apical third of curved roots. CUI has been reported to have a significant higher efficacy in removing pulp tissue and debris from root canal system than PUI and CSNI (Jiang et al. 2012; Neelakantan et al. 2016). Malentacca *et al.* (Malentacca et al. 2012) compared the efficacy and safety of CUI, PUI, ANP and CSNI in vitro study and found CUI was the most effective system while ANP was the safest, but only by a slight margin compared with CUI, which was reported to be the best to reconcile efficacy and safety among these four irrigation systems.

#### **1.5.2.5 Laser Activated Irrigation (LAI) and Photon-Induced Photoacoustic Streaming (PIPS)**

LIA is a method which uses laser to “activate” the liquid. The liquid vaporizes and expands to form bubbles. When the laser pulse ceases, bubbles begin to shrink and result in acoustic waves which aid in cleaning the root canal by shearing debris off the walls (Matsumoto et al. 2011). PIPS (Fidelis, Fotona, Ljubljana, Slovenia) is a special form of LIA that works indirectly and without the thermal effect by activating irrigants. It creates a strong photoacoustic shockwave that streams

irrigants throughout the root canal system. Unlike other conventional laser applications, the PIPS tip is not placed inside the canal system but in the pulp chamber. Peters et al. (2011) compared the disinfection and disruption of biofilm within the root canal in the apical third and found PIPS generated more negative samples and removed biofilm better than PUI, but was not able to completely remove the biofilm. LIA / PIPS have also been reported to be more effective in debris removal than PUI (de Groot et al. 2009; Arslan et al. 2014). One recent study on irrigation safety reported that there was no extrusion of root canal irrigants during the use of LIA / PIPS (Peeters and Mooduto 2013).

#### **1.5.2.6 GentleWave System**

GentleWave System (Sonendo Inc, Laguna Hills, CA, USA) is a novel type of endodontic device developed for root canal cleaning and disinfection (Haapasalo et al. 2014; Ma et al. 2015; Molina et al. 2015; Wohlgemuth et al. 2015; Charara et al. 2016). It uses high-speed fluid dynamics to deliver the irrigants into the root canal system without requiring the tip of the instrument to enter the root canals. Instrumentation can therefore be minimized, which can positively contribute to maintaining strong tooth structure. A few studies have reported its superior soft-tissue dissolution compared to ultrasound and excellent cleaning efficacy in the root canal system (Haapasalo et al. 2014; Ma et al. 2015; Molina et al. 2015). An in vitro study of tissue dissolution by GW and several other irrigation systems showed eight (8) times faster soft tissue dissolution by GW than by any other system studied (Haapasalo et al. 2014). GW, when used with sterile water only dissolved tissue comparable to PUI with 2% NaOCl, giving basis for speculation that the high speed irrigant flow hitting the end plate of the nozzle of the GW cleaning instrument creates a strong cavitation effect. Further, all irrigants in the GW system are degassed in real time just before entering the

pulp chamber and root canal system, which might explain the spread of the cavitation effect much farther away from the instrument tip than with “conventional” ultrasound used with solutions which are not degassed (Haapasalo et al. 2014).

Two clinical studies have reported a high level of success for cases treated by GentleWave System (Sigurdsson et al. 2016; Sigurdsson et al. 2016). So far, there are no studies of the effectiveness of the GentleWave system to remove bacteria from the root canals.

### **1.5.3 Fluid Hydrodynamics**

Irrigation dynamics is important (Moser and Heuer 1982) for the effectiveness of irrigation. This depends on the mechanism(s) of action of the irrigant and on how the irrigant can be brought in contact with the microorganisms and tissue debris (Chow 1983). Smear layer and biofilm are removed by chemical action and physical shear stress by the irrigant on the canal wall. Wall shear stress is difficult to measure. It depends on the flow velocity gradient in closest contact with the wall. Computational fluid dynamics (CFD) is a branch of research focusing on the various phenomena in irrigant flow at different environments. CFD studies have evaluated the effect of root canal taper and apical preparation size on irrigant flow inside a root canal during final irrigation (Boutsioukis et al. 2010a; Boutsioukis et al. 2010b). Higher taper particularly in the apical canal improves irrigant replacement and wall shear stress and reduces apical pressure and extrusion of the irrigant. An irrigant in a non- or minimally tapered root canal with a large apical preparation also showed better irrigant replacement and wall shear stress than in canals with a small preparation size. Irrigant velocity is very important for the overall effectiveness of irrigation (Fariniuk et al. 2003). Previous studies have showed that the irrigant in the canal is only reaching

1-3 mm beyond the tip of the needle in simulated curved canals. In order to obtain total replacement of an irrigating solution in the root canal, the irrigation needle should be placed at close proximity of the apical foramen (Park et al. 2013). However, this also increases the risk for irrigant extrusion, which in particular with NaOCl should be avoided at all cost. In recent years studies using CFD models to examine irrigant flow have shed light to many poorly understood areas in root canal irrigation and helped to increase the evaluation of risk for irrigant extrusion (Boutsioukis et al. 2010; Shen et al. 2010; Snjaric et al. 2012).

## **1.6 Methods of Sampling Bacteria from Root Canals**

In the process of studying the composition of the root canal microbiota and in quantifying the endodontic microbes, the major challenge is adequate and representative sampling of the complete root canal system. Sampling of the microbes has been done with paper points, endodontic files, or by aspirating the sample fluid in the root canal (Berber et al. 2006; Harrison et al. 2010; Huffaker et al. 2010). The root canal anatomy makes it impossible for a paper point or file to touch all areas of the root canal system (Sathorn et al. 2007). These sampling methods are best suited for planktonic bacteria and those only loosely attached to biofilm. With regard to biofilms, sampling with paper points is unlikely to effectively detach and collect bacteria from them. Further, these sampling methods only address areas where instrumentation has created access. Many areas are simply left untouched by the paper point or file. To increase the yield, agitation of the sample fluid by sonic or ultrasonic equipment is recommended (Harrison et al. 2010; Huffaker et al. 2010; Grundling et al. 2011). However, even then the impact on biofilm is uncertain. In some studies, the whole dentin block or root which has been cut off the crown after extraction, has been frozen and pulverized to maximize the numbers of bacteria for the culture (Kho and Baumgartner 2006;



Miller and Baumgartner 2010). A study that compared the efficacy of pulverization and sterile paper point for sampling root canals concluded that the pulverization was more effective than paper point sampling. However, pulverization is an invasive and destructive method which may bring contamination e.g. from the root surface.

## **1.7 Quantification of the Biofilm Microbes**

### **1.7.1 Culture Based Methods**

Culture-based methods have traditionally been used to identify and quantify bacteria in different infections (Chu et al. 2005; Martinho et al. 2015). Counting of colony-forming units (CFUs) on selective or nonselective agar culture plates is a technique which has been used to quantify microorganisms for more than a century. However, some bacteria are difficult to grow because they have specific requirements for nutrients and around one-third of oral bacteria cannot be cultured using conventional methods (Conrads et al. 1997; Wade et al. 2016). Furthermore, low sensitivity and long time required are also limitations for the conventional methods (Siqueira and Rocas 2005).

### **1.7.2 qPCR**

To overcome the limitations of culture-based detection methods and analyses, molecular methods have been applied to detect and quantify microbes. Quantitative Real-time Polymerase Chain Reaction (qPCR) is a technique of molecular biology based on the polymerase chain reaction (PCR). It can monitor the amplification of DNA products not just at its end as in conventional PCR, but in real-time during the run. As early as 2001, real-time qPCR was used to

detect and quantify the number of bacteria in oral plaque samples (Sakamoto et al. 2001). qPCR is increasingly used in microbiological investigations because of its high sensitivity and efficiency in both in vitro and in vivo studies to quantify specific species or e.g. the whole bacterial load in polymicrobial infections (Fouad et al. 2002; Kim et al. 2013; Antunes et al. 2015; Rocas et al. 2015; Rodrigues et al. 2015). qPCR can detect DNA already from 10 copies, thus being much more sensitive than culture method, which can only quantify culturable organisms when they are present in high enough numbers (Araujo et al. 2015). Studies which have compared the qPCR and conventional culture method with their abilities to quantify bacteria concluded that qPCR offered significant advantages regarding speed and sensitivity of detection (Boutaga et al. 2003; Sedgley et al. 2006).

Some recent studies reported DNA had a strong binding affinity to dentin and hydroxyapatite (Brundin et al. 2013; Brundin et al. 2014). The resultant dentin-bound DNA preserved DNA against natural decomposition and protected DNA against nuclease activity in bacterial culture. Excreted extracellular DNA is a natural part of biofilm development and growth (Whitchurch et al. 2002; Martins et al. 2010; Barnes et al. 2012). The contact of extracellular DNA with dentin in infected root canal may well increase the amount of preserved bacterial DNA. The preserved DNA can be recovered when DNA samples are taken from the root canals.

## **1.8 Aim of the Study**

The aim of this study was to compare the in vitro effectiveness of removal of bacterial load from infected root canals, as measured by qPCR, by GentleWave System and an Ultrasonic System. Total bacterial load, as well as the amount of *E. faecalis* and *Streptococcus* species were

determined before instrumentation, after instrumentation and after the two different energy intensive treatments.

## **1.9 Hypothesis**

The null hypothesis is: There is no significant difference between GentleWave System and ProUltra PiezoFlow Active Ultrasonic System in their effectiveness of removing bacteria from infected root canals in vitro.

## **Chapter 2: Materials and Methods**

### **2.1 Tooth Collection and Preparation**

Ethics permission was obtained from the University of British Columbia Office of Research Services, Clinical Research Ethics Board (certificate number H15-02793). Sample size was calculated based on the pilot study with power of 0.95 using statistical software (G\*Power 3.1.9.2, Heinrich Heine, University of Dusseldorf). Fifteen extracted human molars were collected and stored in PBS until use. Any teeth with decay or fractures below the cemento-enamel junction, internal or external resorption, open apices, or previous root canal therapy were excluded.

Endodontic access was achieved as per standard practice, and patency was confirmed utilizing a #10 K-file. Working length (WL) was defined as 1 mm from the radiographic apex. The teeth were instrumented to #15/.02 and then cleaned with the GentleWave System to provide the same baseline for all the teeth. Samples were submerged in 10 mL of PBS and autoclaved for sterility at 121°C for 25 minutes. The apices of the roots of all the teeth were sealed using hot glue.

### **2.2 Inoculation**

The supragingival and subgingival plaque was collected from interdental spaces of molar teeth of an adult volunteer using sterile wooden sticks. The plaque was suspended in BHI and incubated anaerobically at 37°C for two days. Individual molars were filled with approximately 120 µL of mixed plaque in BHI suspension. Specimens were centrifuged at 3500 g for 5 minutes. The process was repeated 3 times with a fresh solution of bacteria during each centrifugation. All the teeth were incubated in BHI broth for 2 weeks at 37°C in air.

### **2.3 Sampling of the Bacteria from the Canals, First Sample**

After two weeks of incubation, samples were divided randomly into two treatment groups. The initial sample (S1) was taken before treatment. Teeth were taken out from the BHI broth by using sterile tweezers, the outer surface of the teeth was first carefully wiped with CaviWipes (Metrex Research, Orange, CA, U.S.A) to clean and disinfect the teeth surface. Pulp chamber was carefully dried with sterile cotton pellets without affecting the canals. The samples were taken by sterile paper points (#15, Diadent Group International, Seoul, Korea) inserted into each root canal without touching the wall of the pulp chamber. Several paper points were used until the fluid in the root canal was soaked and the root canal appeared dry. Samples from each canal were collected into separate, sterile 1 mL Eppendorf tubes filled with PBS and frozen at -20°C until used.

### **2.4 GentleWave Group, Second and Third Sample**

Seven teeth were allocated into GentleWave group. A #10 (or smaller) hand K-file was inserted into the canals to measure the WL (apical foramen minus 1 mm). Pulp chamber was filled with sterile water and the canals were instrumented with #15 hand K-file (Dentsply Tulsa, Tulsa, OK, U.S.A) to WL followed by Vortex Blue™ Rotary #15/.04 files at 350 rpm. One mL of sterile water was used to irrigate the canals with a 5mL syringe and a 30G side vented needle after the hand and rotary file. The irrigation needle was placed as deep as possible without binding, but not closer than 1 mm from the WL. A #10 hand K-file was employed to verify that WL could be reached after finished instrumentation. Pulp chamber was dried as described above and a second bacteriological sample (S2) was taken from the canals with sterile paper points as above and frozen. The teeth were then treated using the GentleWave System, according to the following

protocol: 3 min irrigation using 3% NaOCl, sterile water for 30 sec, 8% EDTA for 2 min. Final irrigation was with sterile water for 15 sec. The pulp chamber was dried as above, and the third sample (S3) was taken from each canal and frozen for later analysis.

## **2.5 Ultrasonic System Group, Second and Third Sample**

Eight teeth were divided into Ultrasonic System group. A #10 (or smaller) hand K-file was used to measure WL. Pulp chamber was filled with sterile water and the canals were instrumented with #15 and #20 hand K-file to WL. Vortex Blue Rotary 04 taper files were then used at 350rpm in the following order: #15, #20, #25, #30 and #35, all to WL. One mL of sterile water was used to irrigate the canals between each file. After instrumentation, a second sample (S2) was taken as described above. Final irrigation was performed with the ProUltra PiezoFlow Active Ultrasonic System as follows: 3% NaOCl 1 min per canal, sterile water 10 sec per canal, 8% EDTA 1 min per canal and sterile water 10 sec per canal. After the ultrasonic treatment, the pulp chamber was dried and the final sample (S3) was taken as described above.

## **2.6 DNA Extraction and Quantitative Real-time PCR Analysis**

The frozen samples were thawed to room temperature, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer. The final volume of DNA solution of each sample was 150  $\mu$ L and was taken into account during calculation.

The total bacterial load was quantified by using 16S ribosomal RNA gene-targeted qPCR. The levels of *Enterococcus faecalis* and *Streptococcus* species (genus specific primer) were evaluated

by using specific primers for *E. faecalis* and *Streptococcus* species. Table 1 shows the sequences and annealing temperatures of each primer. The qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA). The total reaction volume was 20  $\mu$ L. Each reaction included 10  $\mu$ L of Power SYBR Green PCR Master Mix, 6  $\mu$ L of sterile distilled water, 1  $\mu$ L of each 10  $\mu$ M primers and 2  $\mu$ L DNA template. The cycling conditions for universal bacteria and *Streptococcus* species contained 10 min at 95°C followed by 40 repeats of 95°C for 1 min, annealing for 1 min (temperatures shown in Table 2.1), and 72°C for 1 min. The temperature setting for *E. faecalis* was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Triplicate measurements were done for all samples. Each measurement included triplicate negative controls which contained all reactants but no template DNA. Melt curve analysis was performed after amplification to confirm the specificity of the amplified reaction. The melt curve was detected from 60°C to 95°C. Fluorescence measurements were taken continuously at every 1% increase in temperature. StepOne Software v2.3 (Applied Biosystems) was used to acquire and analyze the data.

Standard curves were constructed by 10-fold diluted DNA extracted from *E. faecalis* ATCC 29212 and *Streptococcus mutans* ATCC 25175. The concentration of the pure extracted DNA was quantified using GeneQuant™ pro RNA/DNA Calculator (GE Healthcare, Little Chalfont, UK). Genome copy levels were calculated using the formula  $m = n(1 \text{ mole}/6 \times 10^{23} [\text{bp}])(660 [\text{g}]/\text{mole}) = n(1.096 \times 10^{-21} [\text{g}/\text{bp}])$  (Antunes et al. 2015), where m is the genomic mass of a single cell and n is the genome size. *Streptococcus mutans* ATCC 25175 was also used for total

bacteria quantification because it contained 5 copies of 16S rRNA gene and 5 is the approximate average number of 16S rRNA genes which most oral bacteria have (Rocas et al. 2015).



**TABLE 2.1** Primers Used in Real-time PCR for Bacterial Quantification

Target	Primer sequence	Annealing temp (°C)	Reference
Universal 16S rRNA gene	5'-CAD ACT CCT ACG GGA GGC-3'	59	(Dorn-In et al. 2015)
	5'-ATC CTG TTT GMT MCC CVC RC-3'		
<i>Enterococcus faecalis</i> *	5'-CAA ACT GTT GGC ATT CCA CAA-3'	60	
	5'-TGG ATT TCC TTT CCA GTC ACT TC-3'		
<i>Streptococcus</i> species	5'-AGA GTT TGA TYM TGG CTC AG-3'	58	(Rocas and Siqueira 2012)
	5'-TTA GCC GTC CCT TTC TGG T-3'		

\* Another primer for *E. faecalis* previously used in (Rocas and Siqueira 2012) was tried in pilot experiments, but the results showed variation and indicated of less than optimal performance. Therefore, a new primer sequence was chosen based on the analysis of Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and preliminary testing.

## 2.7 Statistical Analysis

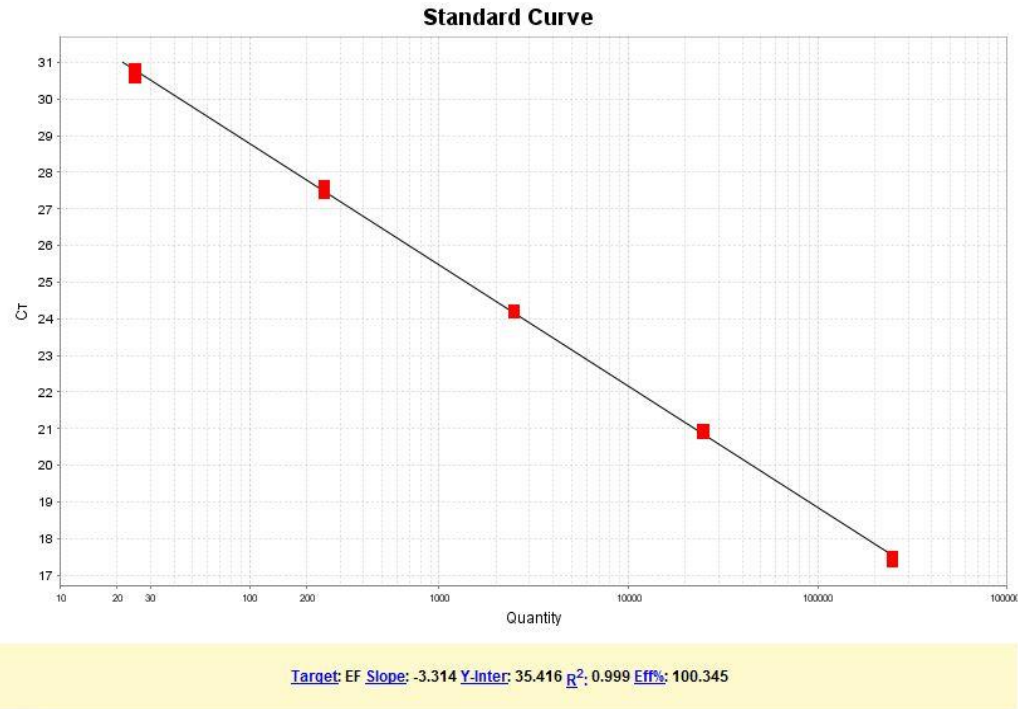
Statistical analysis was performed with SPSS 16.0 software (SPSS Inc, Chicago, IL). The bacterial reduction from S1 to S2 as well as S1 to S3 were compared between two systems for total bacteria, *E. faecalis* and *Streptococcus* species, respectively by using Mann-Whitney U test. The significance level was set at  $P < 0.05$ .

## Chapter 3: Results

### 3.1 qPCR Co-efficiency Test and Specificity

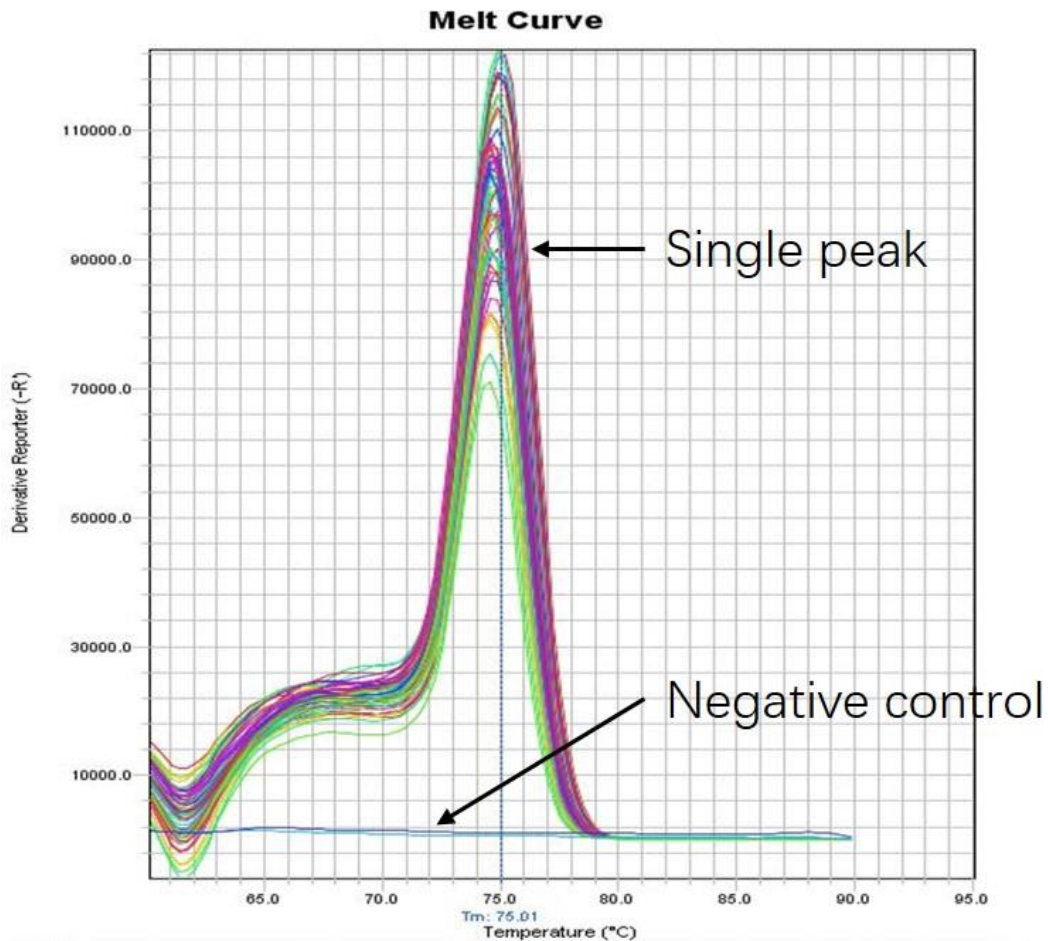
A total of 43 canals were included in this study (17 canals for Gentlewave group and 26 canals for PF ultrasonic group). Three samples (S1, S2, S3) were taken from each canal. There were 129 samples evaluated by qPCR. All runs were taken in triplicate for samples and standards. Standard curve of qPCR Co-efficiency test was used to evaluate the amplification efficiency of each run (Figure 3.1). Purified DNA from standard strains was diluted from  $10^5$  copies/ $\mu\text{L}$  to 10 copies/ $\mu\text{L}$  to construct the standard curve. The Co-efficiency was calculated based on the slope of the standard curve by using the formula:  $\text{Efficiency} = 10^{(-1/\text{slope})} - 1$ . In this study, the average Co-efficiencies for Universal, *E. faecalis* and *Streptococcus* species were 94.67%, 98.65% and 92.59%, respectively. Co-efficiency for each run was within the normal range of the Co-efficiency for all qPCR assays (90%-110%).

The regression coefficient was calculated from the regression line in the standard curve. The  $R^2$  value indicated the closeness of fit between the standard curve regression line and the individual Ct data points from the standard reaction. A value of 1 indicated a perfect fit between the regression line and the data points. In this study, the average  $R^2$  values of all runs for Universal, *E. faecalis* and *Streptococcus* species were 0.992, 0.999, and 0.996, respectively, which illustrated a high correlation between the bacterial copy counts and Ct values (Figure 3.1).



**Figure 3.1. Standard Curve of qPCR Co-efficiency Test. The average Co-efficiencies for Universal, *E. faecalis* and *Streptococcus* species were 94.67%, 98.65% and 92.59%. R<sup>2</sup> values of all runs for Universal, *E. faecalis* and *Streptococcus* species were 0.992, 0.999, and 0.996.**

Melt curve of each qPCR assay was used to confirm the specificity of the primers. In this study, melt curve with a single peak and no amplification in negative control were detected in every qPCR assay, which indicated a high specificity of the primers (Figure 3.2).



**Figure 3.2. Melt Curve of qPCR Assay - qPCR Specificity. Melt curve with a single peak and no amplification in negative control was detected in every qPCR assay, which indicated a high specificity of the primers.**

### **3.2 qPCR Results of Bacterial Quantification**

Both treatment groups presented a high reduction of bacterial numbers from S1 to S3, as calculated from the number of DNA copies. The reduction by GentleWave System was greater than by the Ultrasonic System in all three bacterial groups (Table 3.1-3.3). In GentleWave System group, a mean number of  $4.28 \times 10^7$  total bacterial cell equivalents per canal were detected in S1 samples and significantly decreased in S3 to a mean of  $5.58 \times 10^4$  cells per canal (P

< 0.001); the mean reduction in total bacterial counts was 99.85% with the range from 99.27% to 99.99%. In Ultrasonic System group, the reduction in the mean number of bacteria from S1 to S3 was from  $1.84 \times 10^7$  to  $5.13 \times 10^4$  cells per canal, or 97.96%, with individual canals ranging from 85.77% to 99.99%. The difference between GentleWave System and the Ultrasonic System was statistically significant ( $P = 0.004$ ).

*E. faecalis* was observed at a mean value of  $6.28 \times 10^5$  before instrumentation and  $1.35 \times 10^3$  after treatment in GentleWave System group. The mean reduction (S1 to S3) was 99.73% by GentleWave and 97.18% by the Ultrasound ( $P = 0.008$ ). Table 3.2 shows the mean numbers of *E. faecalis* per canal and the mean reduction percentages for the two treatment groups.

Slightly smaller but still statistically significant difference between the two cleaning methods was measured in the *Streptococcus* group (genus specific primer), 99.61% for GentleWave and 98.53% for the Ultrasonic System ( $P = 0.028$ ) (Table 4).

There was no significant difference in the decrease of the microbes between the two groups after mechanical instrumentation only (S1 to S2) ( $P > 0.05$ ) (Tables 3.1-3.3).

**TABLE 3.1** Level of Total Bacteria in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave / PF Ultrasonic System (S3) (Mean  $\pm$  Standard error). The difference of reduction% from S1 to S3 between GentleWave System and the Ultrasonic System was statistically significant (P = 0.004)

Groups	N (Canals)	Mean (copies/canal) $\pm$ SE			Reduction% S1 to S2	Reduction% S1 to S3	
		S1	S2	S3	Mean $\pm$ SE	Mean $\pm$ SE	Range
GentleWave	17	$4.14 \times 10^7 \pm 9.26 \times 10^6$	$8.58 \times 10^6 \pm 4.23 \times 10^6$	$5.48 \times 10^4 \pm 1.57 \times 10^4$	85.55% $\pm$ 3.16%	99.85% $\pm$ 0.04%	99.27% - 99.99%
PF Ultrasonic	26	$1.85 \times 10^7 \pm 4.41 \times 10^6$	$4.17 \times 10^6 \pm 1.54 \times 10^6$	$5.97 \times 10^4 \pm 1.90 \times 10^4$	81.70% $\pm$ 2.20%	97.96% $\pm$ 0.71%	85.77% - 99.99%

**TABLE 3.2** Level of *Enterococcus faecalis* in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave / PF Ultrasonic System (S3) (Mean  $\pm$  Standard error). The difference of reduction% from S1 to S3 between GentleWave System and the Ultrasonic System was statistically significant (P = 0.008)

Groups	N (Canals)	Mean (copies/canal) $\pm$ SE			Reduction % S1 to S2	Reduction% S1 to S3	
		S1	S2	S3	Mean $\pm$ SE	Mean $\pm$ SE	Range
GentleWave	17	$6.28 \times 10^5 \pm 1.70 \times 10^5$	$1.56 \times 10^5 \pm 3.60 \times 10^4$	$1.35 \times 10^3 \pm 3.30 \times 10^2$	73.73% $\pm$ 4.69%	99.73% $\pm$ 0.08%	99.19% - 99.99%
PF Ultrasonic	26	$3.39 \times 10^5 \pm 1.11 \times 10^5$	$8.63 \times 10^4 \pm 1.72 \times 10^4$	$1.79 \times 10^3 \pm 5.50 \times 10^2$	66.59% $\pm$ 4.85%	97.18% $\pm$ 0.83%	84.21% - 99.99%



**TABLE 3.3** Level of *Streptococcus* species in Root Canal Samples of Teeth Taken Before Instrumentation (S1), After Instrumentation (S2) and After GentleWave / PF Ultrasonic System (S3) (Mean  $\pm$  Standard error). The difference of reduction% from S1 to S3 between GentleWave System and the Ultrasonic System was statistically significant (P = 0.028)

Groups	N (Canals)	Mean (copies/canal) $\pm$ SE			Reduction % S1 to S2	Reduction% S1 to S3	
		S1	S2	S3	Mean $\pm$ SE	Mean $\pm$ SE	Range
<b>GentleWave</b>	17	$3.10 \times 10^4 \pm 1.36 \times 10^4$	$4.74 \times 10^3 \pm 9.71 \times 10^2$	$3.15 \times 10^1 \pm 9.46 \times 10^0$	65.63% $\pm$ 5.40%	99.61% $\pm$ 0.21%	96.31% - 99.99%
<b>PF Ultrasonic</b>	26	$1.02 \times 10^4 \pm 2.95 \times 10^3$	$2.68 \times 10^3 \pm 6.39 \times 10^2$	$4.05 \times 10^1 \pm 8.86 \times 10^0$	64.69% $\pm$ 5.13%	98.53% $\pm$ 0.58%	86.02% - 99.99%

## Chapter 4: Discussion

Treatment with both GentleWave multisonic and ProUltra PiezoFlow ultrasonic systems greatly reduced the bacterial load in the canals. The average reduction of total bacteria, streptococci and *E. faecalis* was 99.61 – 99.85 % in the GW group and 97.18 - 98.53 % in the ultrasound group. There were no differences in bacterial reduction between canals from teeth with two, three or four canals (data not shown). The change in bacterial counts in the three measured bacterial groups (total bacteria, *E. faecalis*, streptococci) from S1 (start) to S2 (after mechanical instrumentation), before cleaning using the two energy-driven systems was not significantly different between the two groups (Table 3.1- 3.3).

When the results are evaluated based on the magnitude of reduction in bacterial DNA, the differences between the average reductions in each group may seem relatively small, although statistically significant. However, from the point of view of elimination of the endodontic infection and healing of the periapical lesion, it is the number of residual bacteria that is likely to be more important rather than the mere amount of reduction in numbers. In canals cleaned with the GW system, an average of 0.15% of the original flora was still found. In PF treated canals the average residual flora was 2.04% of the original flora. When these numbers are compared between the two groups, it shows that more than ten times as many bacteria are expected to be found in PF cleaned than in GW cleaned canals.

So far, no treatment strategy, cleaning method or antibacterial agent and medicament have been able to predictably provide sterile canals at the end of the chemomechanical treatment, before the root canals are filled. While the results of this study cannot be directly compared to earlier studies,

particularly those where bacterial culture has been used to quantitate the number of bacteria, the results do indicate that the GW cleaning is moving the bar closer to ideal (no bacteria) than previously obtainable. Recent, short term healing results one year after the root canal treatment using GW cleaning showed very high success rates (Sigurdsson et al. 2016), which might be explained at least partly by the excellent results of the present qPCR study of bacterial elimination. However, it is important to emphasize that more long term in vivo clinical studies are needed on healing of apical periodontitis after GW treatment. Also, the results of the present study must be confirmed by others, both by ex vivo and in vivo studies.

While the overall performance of each cleaning system, i.e. the average reduction of microbiota in the root canal system is important, there is another factor which is at least equally important. The range of reduction of total bacterial counts in the individual canals in the GW group was much smaller (99.27% - 99.99%) than in the PF group (85.77% - 99.99%). The result indicates that GW much more predictably achieved a high level of removal of microbes from the root canal system. There is a growing consensus in endodontics that while complete sterility may not be achievable, there is a threshold value for the number of residual microbes, below which healing will occur. It is also possible or even likely that this value is different in individual cases. Based on this kind of hypothesis one can speculate that the high predictability of bacterial reduction by the GW system (very low range and standard deviation), increases the probability that the strong effect brings the residual flora below the threshold value, more often than e.g. with the PF system. It is clear that it is difficult or impossible to make a study designed to directly determine the threshold value of bacteria for predictable healing. However, future in vivo studies of healing combined with analysis

of the residual microflora with sensitive molecular methods, may give indirect information on this topic.

One of the main benefits of *in vitro* studies is that it is usually possible to standardize the experimental conditions fairly well for different groups and thereby minimize the effect of confounding factors. In the present study, this was not possible to the same extent as in many other *in vitro* studies because of the different type of action of the systems as well as recommendations for use by the manufacturers (Zhu et al. 2013; Al Shahrani et al. 2014). The PF system is supposed to be used after conventional size instrumentation (#35/.04 in this study), which allows the tip to be placed freely in the coronal third of the canal (Castelo-Baz et al. 2016). To secure maximal efficacy, the tip must avoid contact with canal walls. The GW system, on the other hand, is designed to work on minimally or even uninstrumented canals, as instead of being inserted into the canal the tip of the instrument is placed in the pulp chamber, just above the chamber floor throughout the treatment. For this reason, the canal dimensions between the groups or within the GW group were not standardized. Another key difference is that PF is used in each canal separately, whereas GW circulates the irrigant simultaneously in all root canals. In other words, PF system provides active ultrasonic cleaning in one canal while the other canals at the same time are more passively exposed to the irrigant. With GW, all canals are actively targeted all the time. The experimental design in the present study was made to conform with the recommended use of the systems rather than testing the ultimate cleaning power of the two in identical conditions, i.e. same canal size and same active time of cleaning per canal facilitated by the multisonic and ultrasonic energy. Therefore, conclusions from the results must be drawn with particular caution. The NaOCl irrigation per tooth was limited to 3 min with GW and 3 or 4 min with PF. If the active ultrasonic

(PF) irrigation with NaOCl had been 3 min per canal, the total NaOCl irrigation per a molar in the present study would have been 9 or 12 minutes, depending on the number of canals (3 or 4), instead of the 3 or 4 minutes now used. Nine to twelve-minute ultrasonic irrigation is not realistic in any clinical situation, in addition, would also come the time for water and EDTA irrigation, multiplied by the number of canals. Corresponding studies with GW and other cleaning and treatment methods in the future are likely to set the focus in different ways, which may result in different experimental designs.

Sampling was done with sterile paper points from the root canals. It is generally accepted that paper point sampling is not the most effective way of collecting all microbes from the root canal system (Antunes et al. 2015). Cutting off the roots and pulverizing them for DNA extraction is likely to yield more bacteria in the samples (Tran et al. 2013). However, when whole roots are sampled, microbes and even residual DNA from root surface, contaminated e.g. during extraction, handling and incubating the root canal microbiota would increase the risk for an error. The exterior apical third of the roots in the present study was sealed with hot glue to prevent the transfer of microbes between the root surface and the root canal system. Another point worth noting is the different volume of the canals in the two groups. One can argue that from a larger canal more bacteria can be picked than from a narrow canal. It is also possible that contrary to the previous claim removal of more dentin by instruments in the PF group mechanically removed the bacteria-rich zones around the main canal, which was the sampling area, and improved the effectiveness of the PF system due to better flow of the irrigants. Nevertheless, the canal dimensions in both groups in samples S2 and S3 remained constant throughout the experiment, and the changes (reduction) in bacterial counts were calculated within each group.

Previous histological studies have indicated that both PF and GW effectively clean hard to reach areas in the root canal system from organic matter (Molina et al. 2015). A micro-CT study with molar canals filled with calcium hydroxide (CH) showed that GW was the only system studied which completely removed the CH paste even from lateral canals and isthmus areas in teeth instrumented to conventional preparation sizes (Ma et al. 2015). PUI was one of the methods in this and other studies that was not able to remove all calcium hydroxide. So far, PF has not been examined in a corresponding setting. The present study showed that GW removed bacteria at a high level of predictability in minimally (#15/.04) instrumented molar canals. The reduction in the amount of total bacterial DNA was in every case at least 99.27%. This is an important finding as effective disinfection is one of the key requirements for minimal instrumentation in endodontics to claim sound scientific basis. Preservation of root dentin can be supposed to contribute to maintaining stronger root and tooth structure than previously possible with conventional instrumentation sizes. Other studies are needed to address technologies and quality of root fillings in minimally instrumented root canals, another key requirement.

## **Chapter 5: Limitations of the Study**

In the present study, sampling was done by paper points, which was considered to be the most suitable method for this study. However, paper point sampling may not optimally reflect the microbiological status in the whole root canal system, especially in the isthmuses and lateral canals. A previous micro-CT study has shown that GW was the only system tested which completely removed a calcium hydroxide paste even from lateral canals and isthmus areas in all molar teeth examined (Ma et al. 2015). It is therefore logical to speculate that GW has the potential to eliminate the microbes in areas unreachable for paper points in root canal system, and bring residual bacteria from the peripheral areas to the main canal, where they may be sampled with the paper points.

The binding affinity of DNA to dentin is another potential concern. Since the present study employed a quantitative qPCR method, the DNA preserved earlier in the root dentin could be recovered when the samples were taken from the root canals. Therefore, this is a factor that should be taken into consideration when designing sample taking procedures for qPCR analysis. Sampling with paper points can minimize the amount of extracellular DNA from dentin as the points cannot penetrate into dentin, whereas dentin/root pulverizing method would include all dentin bound DNA in the sample. Variation in canal anatomy and different length of the roots may also cause differences in the sample volume. However, S1, S2 and S3 of one tooth were taken within the same canal each and compared to each other. Thus, reduction in DNA/bacterial counts were calculated each time within one tooth.

## **Chapter 6: Conclusion**

In conclusion, within the limitation of this ex vivo qPCR study, both PiezoFlow ultrasonic and GentleWave systems effectively reduced the level of bacteria in the root canals of molar teeth. GentleWave System showed more predictably a constantly higher level (> 99%) of bacterial reduction than the Ultrasonic System. Additional in vivo studies are also needed for further investigations into the antibacterial effectiveness of the PiezoFlow Ultrasonic and the GentleWave Systems in the treatment of endodontic infections.



## **Chapter 7: Future Directions**

qPCR is a sensitive and rapid technique to quantify microbes. However, its main limitation is its inability to discriminate between live and dead cells. A method using propidium monoazide (PMA) together with qPCR has been developed to overcome this problem, with reportedly promising results for different bacterial species in different types of samples (Alvarez et al. 2013). In the present study, the effectiveness of removing bacteria (live and dead) was evaluated. A future direction may be using qPCR to detect and compare the ability of different techniques / irrigants in removing and killing microbes.

GW is designed to work on minimally or even uninstrumented canals. Thus, it offers a potentially large clinical advantage over the PiezoFlow method which requires greater removal of root dentin. In this ex vivo study, GW has shown a predictably and constantly high level (> 99%) of bacterial reduction. In the future, the effectiveness of GW should be evaluated in an in vivo study.

## References

- Aderhold, L., H. Knothe and G. Frenkel (1981). "The Bacteriology of Dentogenous Pyogenic Infections." *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **52**: 583-7.
- Al Shahrani, M., E. DiVito, C. V. Hughes, D. Nathanson and G. T. Huang (2014). "Enhanced removal of *Enterococcus faecalis* biofilms in the root canal using sodium hypochlorite plus photon-induced photoacoustic streaming: an in vitro study." *Photomedicine and laser surgery* **32**: 260-6.
- Alvarez, G., M. Gonzalez, S. Isabal, V. Blanc and R. Leon (2013). "Method to quantify live and dead cells in multi-species oral biofilm by real-time PCR with propidium monoazide." *Amb Express* **3**.
- Antunes, H. S., I. N. Rocas, F. R. Alves and J. F. Siqueira, Jr. (2015). "Total and Specific Bacterial Levels in the Apical Root Canal System of Teeth with Post-treatment Apical Periodontitis." *Journal of endodontics* **41**: 1037-42.
- Araujo, P. V., F. Correia-Silva Jde, R. S. Gomez, L. Massara Mde, M. E. Cortes and L. T. Poletto (2015). "Antimicrobial effect of photodynamic therapy in carious lesions in vivo, using culture and real-time PCR methods." *Photodiagnosis and photodynamic therapy* **12**: 401-7.
- Arias-Moliz, M. T., C. M. Ferrer-Luque, M. P. Gonzalez-Rodriguez, M. J. Valderrama and P. Baca (2010). "Eradication of *Enterococcus faecalis* Biofilms by Cetrimide and Chlorhexidine." *J Endod* **36**: 87-90.

- Arslan, H., I. D. Capar, G. Saygili, T. Gok and M. Akcay (2014). "Effect of photon-initiated photoacoustic streaming on removal of apically placed dentinal debris." *Int Endod J* **47**: 1072-7.
- Barnes, A. M., K. S. Ballering, R. S. Leibman, C. L. Wells and G. M. Dunny (2012). "Enterococcus faecalis produces abundant extracellular structures containing DNA in the absence of cell lysis during early biofilm formation." *mBio* **3**: e00193-12.
- Baugh, D. and J. Wallace (2005). "The role of apical instrumentation in root canal treatment: a review of the literature." *Journal of endodontics* **31**: 333-40.
- Berber, V. B., B. P. F. A. Gomes, N. T. Sena, M. E. Vianna, C. C. R. Ferraz, A. A. Zaia and F. J. Souza (2006). "Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing Enterococcus faecalis within root canals and dentinal tubules." *Int Endod J* **39**: 10-7.
- Bergenholtz, G. (1974). "Micro-organisms from necrotic pulp of traumatized teeth." *Odontologisk revy* **25**: 347-58.
- Bergenholtz, G. (1990). "Pathogenic Mechanisms in Pulpal Disease." *J Endod* **16**: 98-101.
- Bhuva, B., S. Patel, R. Wilson, S. Niazi, D. Beighton and F. Mannocci (2010). "The effectiveness of passive ultrasonic irrigation on intraradicular Enterococcus faecalis biofilms in extracted single-rooted human teeth." *Int Endod J* **43**: 241-50.
- Boutaga, K., A. J. van Winkelhoff, C. M. J. E. Vandenbroucke-Grauls and P. H. M. Savelkoul (2003). "Comparison of real-time PCR and culture for detection of Porphyromonas gingivalis in subgingival plaque samples." *J Clin Microbiol* **41**: 4950-4.

- Boutsioukis, C., C. Gogos, B. Verhaagen, M. Versluis, E. Kastrinakis and L. W. M. van der Sluis (2010). "The effect of apical preparation size on irrigant flow in root canals evaluated using an unsteady Computational Fluid Dynamics model." *Int Endod J* **43**: 874-81.
- Boutsioukis, C., C. Gogos, B. Verhaagen, M. Versluis, E. Kastrinakis and L. W. M. van der Sluis (2010). "The effect of root canal taper on the irrigant flow: evaluation using an unsteady Computational Fluid Dynamics model." *Int Endod J* **43**: 909-16.
- Boutsioukis, C., E. Kastrinakis, T. Lambrianidis, B. Verhaagen, M. Versluis and L. W. van der Sluis (2014). "Formation and removal of apical vapor lock during syringe irrigation: a combined experimental and Computational Fluid Dynamics approach." *Int Endod J* **47**: 191-201.
- Boutsioukis, C., B. Verhaagen, M. Versluis, E. Kastrinakis, P. R. Wesselink and L. W. M. van der Sluis (2010). "Evaluation of Irrigant Flow in the Root Canal Using Different Needle Types by an Unsteady Computational Fluid Dynamics Model." *J Endod* **36**: 875-9.
- Boutsioukis, C., B. Verhaagen, A. D. Walmsley, M. Versluis and L. W. van der Sluis (2013). "Measurement and visualization of file-to-wall contact during ultrasonically activated irrigation in simulated canals." *Int Endod J* **46**: 1046-55.
- Brauner, A. W. and G. Conrads (1995). "Studies into the Microbial Spectrum of Apical Periodontitis." *Int Endod J* **28**: 244-8.
- Brook, I., E. H. Frazier and M. E. Gher (1991). "Aerobic and Anaerobic Microbiology of Periapical Abscess." *Oral Microbiol Immun* **6**: 123-5.
- Brundin, M., D. Figdor, A. Johansson and U. Sjogren (2014). "Preservation of bacterial DNA by human dentin." *J Endod* **40**: 241-5.

- Brundin, M., D. Figdor, G. Sundqvist and U. Sjogren (2013). "DNA binding to hydroxyapatite: a potential mechanism for preservation of microbial DNA." *J Endod* **39**: 211-6.
- Cachovan, G., U. Schiffner, S. Altenhof, A. Guentsch, W. Pfister and S. Eick (2013). "Comparative antibacterial efficacies of hydrodynamic and ultrasonic irrigation systems in vitro." *J Endod* **39**: 1171-5.
- Carr, G. B., R. S. Schwartz, C. Schaudinn, A. Gorur and J. W. Costerton (2009). "Ultrastructural examination of failed molar retreatment with secondary apical periodontitis: an examination of endodontic biofilms in an endodontic retreatment failure." *J Endod* **35**: 1303-9.
- Castelo-Baz, P., P. Varela-Patino, G. Cantatore, A. Dominguez-Perez, M. Ruiz-Pinon, R. Miguens-Vila and B. Martin-Biedma (2016). "In vitro comparison of passive and continuous ultrasonic irrigation in curved root canals." *Journal of clinical and experimental dentistry* **8**: e437-e41.
- Charara, K., S. Friedman, A. Sherman, A. Kishen, G. Malkhassian, M. Khakpour and B. Basrani (2016). "Assessment of Apical Extrusion during Root Canal Irrigation with the Novel GentleWave System in a Simulated Apical Environment." *J Endod* **42**: 135-9.
- Chavez de Paz, L. E., G. Bergenholtz, G. Dahlen and G. Svensater (2007). "Response to alkaline stress by root canal bacteria in biofilms." *Int Endod J* **40**: 344-55.
- Chow, T. W. (1983). "Mechanical Effectiveness of Root-Canal Irrigation." *J Endod* **9**: 475-9.
- Chu, F. C., C. S. Tsang, T. W. Chow and L. P. Samaranayake (2005). "Identification of cultivable microorganisms from primary endodontic infections with exposed and unexposed pulp space." *Journal of endodontics* **31**: 424-9.

- Chugal, N. M., J. M. Clive and L. S. Spangberg (2001). "A prognostic model for assessment of the outcome of endodontic treatment: Effect of biologic and diagnostic variables." *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **91**: 342-52.
- Cobankara, F. K., H. B. Ozkan and A. Terlemez (2010). "Comparison of organic tissue dissolution capacities of sodium hypochlorite and chlorine dioxide." *J Endod* **36**: 272-4.
- Conrads, G., S. E. Gharbia, K. Gulabivala, F. Lampert and H. N. Shah (1997). "The use of a 16S rDNA directed PCR for the detection of endodontopathogenic bacteria." *Journal of endodontics* **23**: 433-8.
- Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber and G. James (1994). "Biofilms, the customized microniche." *Journal of bacteriology* **176**: 2137-42.
- Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). "Bacterial biofilms: a common cause of persistent infections." *Science* **284**: 1318-22.
- Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). "Bacterial biofilms: A common cause of persistent infections." *Science* **284**: 1318-22.
- Czonstkowsky, M., E. G. Wilson and F. A. Holstein (1990). "The smear layer in endodontics." *Dental clinics of North America* **34**: 13-25.
- de Gregorio, C., R. Estevez, R. Cisneros, A. Paranjpe and N. Cohenca (2010). "Efficacy of different irrigation and activation systems on the penetration of sodium hypochlorite into simulated lateral canals and up to working length: an in vitro study." *J Endod* **36**: 1216-21.
- de Groot, S. D., B. Verhaagen, M. Versluis, M. K. Wu, P. R. Wesselink and L. W. M. van der Sluis (2009). "Laser-activated irrigation within root canals: cleaning efficacy and flow visualization." *Int Endod J* **42**: 1077-83.

- de Paz, L. E. C. (2012). "Development of a Multispecies Biofilm Community by Four Root Canal Bacteria." *J Endod* **38**: 318-23.
- de Paz, L. E. C., G. Bergenholtz and G. Svensater (2010). "The Effects of Antimicrobials on Endodontic Biofilm Bacteria." *J Endod* **36**: 70-7.
- Dorn-In, S., R. Bassitta, K. Schwaiger, J. Bauer and C. S. Holzel (2015). "Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA." *Journal of microbiological methods* **113**: 50-6.
- Du, T. F., Q. Shi, Y. Shen, Y. G. Cao, J. Z. Ma, X. P. Lu, Z. L. Xiong and M. Haapasalo (2013). "Effect of Modified Nonequilibrium Plasma with Chlorhexidine Digluconate against Endodontic Biofilms In Vitro." *J Endod* **39**: 1438-43.
- Du, T. F., Z. J. Wang, Y. Shen, J. Z. Ma, Y. G. Cao and M. Haapasalo (2014). "Effect of Long-term Exposure to Endodontic Disinfecting Solutions on Young and Old *Enterococcus faecalis* Biofilms in Dentin Canals." *J Endod* **40**: 509-14.
- Du, T. F., Z. J. Wang, Y. Shen, J. Z. Ma, Y. G. Cao and M. Haapasalo (2015). "Combined Antibacterial Effect of Sodium Hypochlorite and Root Canal Sealers against *Enterococcus faecalis* Biofilms in Dentin Canals." *J Endod* **41**: 1294-8.
- Dunavant, T. R., J. D. Regan, G. N. Glickman, E. S. Solomon and A. L. Honeyman (2006). "Comparative evaluation of endodontic irrigants against *Enterococcus faecalis* biofilms." *J Endod* **32**: 527-31.
- Eckerbom, M., J. E. Andersson and T. Magnusson (1989). "A Longitudinal-Study of Changes in Frequency and Technical Standard of Endodontic Treatment in a Swedish Population." *Endodontics & dental traumatology* **5**: 27-31.

- Fariniuk, L. F., F. Baratto, A. M. da Cruz and M. D. de Sousa-Neto (2003). "Histologic analysis of the cleaning capacity of mechanical endodontic instruments activated by the ENDOflash system." *J Endod* **29**: 651-3.
- Figdor, D. and G. Sundqvist (2007). "A big role for the very small - understanding endodontic microbial flora." *Aust Dent J* **52**: S38-S51.
- Fouad, A. F., J. Barry, M. Caimano, M. Clawson, Q. Zhu, R. Carver, K. Hazlett and J. D. Radolf (2002). "PCR-based identification of bacteria associated with endodontic infections." *Journal of clinical microbiology* **40**: 3223-31.
- Frenkel, G. and R. Stellmach (1973). "The treatment of apical periodontitis. Apicoectomy with retrograde root canal filling." *Quintessence international, dental digest* **4**: 17-23.
- Gilbert, P., J. Das and I. Foley (1997). "Biofilm susceptibility to antimicrobials." *Adv Dent Res* **11**: 160-7.
- Gilbert, P., D. J. Evans and M. R. Brown (1993). "Formation and dispersal of bacterial biofilms in vivo and in situ." *The Journal of applied bacteriology* **74 Suppl**: 67S-78S.
- Goldman, M., L. B. Goldman, R. Cavaleri, J. Bogis and P. S. Lin (1982). "The Efficacy of Several Endodontic Irrigating Solutions - a Scanning Electron-Microscopic Study .2." *J Endod* **8**: 487-92.
- Gomes, B. P. F. A., C. C. R. Ferraz, M. E. Vianna, V. B. Berber, F. B. Teixeira and F. J. Souza (2001). "In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*." *Int Endod J* **34**: 424-8.



- Goncalves, L. S., R. C. Rodrigues, C. V. Andrade Junior, R. G. Soares and M. V. Vettore (2016). "The Effect of Sodium Hypochlorite and Chlorhexidine as Irrigant Solutions for Root Canal Disinfection: A Systematic Review of Clinical Trials." *J Endod* **42**: 527-32.
- Grenier, D. and D. Mayrand (1986). "Nutritional Relationships between Oral Bacteria." *Infect Immun* **53**: 616-20.
- Grundling, G. L., J. G. Zechin, W. M. Jardim, S. D. de Oliveira and J. A. P. de Figueiredo (2011). "Effect of Ultrasonics on Enterococcus faecalis Biofilm in a Bovine Tooth Model." *J Endod* **37**: 1128-33.
- Haapasalo, M. and D. Orstavik (1987). "In vitro infection and disinfection of dentinal tubules." *J Dent Res* **66**: 1375-9.
- Haapasalo, M., Y. Shen, Z. Wang and Y. Gao (2014). "Irrigation in endodontics." *British dental journal* **216**: 299-303.
- Haapasalo, M., Z. Wang, Y. Shen, A. Curtis, P. Patel and M. Khakpour (2014). "Tissue dissolution by a novel multisonic ultracleaning system and sodium hypochlorite." *Journal of endodontics* **40**: 1178-81.
- Handley, P. S., P. L. Carter, J. E. Wyatt and L. M. Hesketh (1985). "Surface-Structures (Peritrichous Fibrils and Tufts of Fibrils) Found on Streptococcus-Sanguis Strains May Be Related to Their Ability to Coaggregate with Other Oral Genera." *Infect Immun* **47**: 217-27.
- Harrison, A. J., P. Chivatxaranukul, P. Parashos and H. H. Messer (2010). "The effect of ultrasonically activated irrigation on reduction of Enterococcus faecalis in experimentally infected root canals." *Int Endod J* **43**: 968-77.

- Herrero, E. R., N. Boon, M. Pauwels, K. Bernaerts, V. Slomka, M. Quirynen and W. Teughels (2017). "Necrotrophic growth of periodontopathogens is a novel virulence factor in oral biofilms." *Scientific reports* **7**.
- Huffaker, S. K., K. Safavi, L. S. W. Spangberg and B. Kaufman (2010). "Influence of a Passive Sonic Irrigation System on the Elimination of Bacteria from Root Canal Systems: A Clinical Study." *J Endod* **36**: 1315-8.
- Huque, J., K. Kota, M. Yamaga, M. Iwaku and E. Hoshino (1998). "Bacterial eradication from root dentine by ultrasonic irrigation with sodium hypochlorite." *Int Endod J* **31**: 242-50.
- Jenkinson, H. F. and H. M. Lappin-Scott (2001). "Biofilms adhere to stay." *Trends Microbiol* **9**: 9-10.
- Jhajharia, K., A. Parolia, K. V. Shetty and L. K. Mehta (2015). "Biofilm in endodontics: A review." *Journal of International Society of Preventive & Community Dentistry* **5**: 1-12.
- Jiang, L. M., B. Lak, L. M. Eijssvogels, P. Wesselink and L. W. van der Sluis (2012). "Comparison of the cleaning efficacy of different final irrigation techniques." *J Endod* **38**: 838-41.
- Kahn, F. H., P. A. Rosenberg and J. Gliksberg (1995). "An in vitro evaluation of the irrigating characteristics of ultrasonic and subsonic handpieces and irrigating needles and probes." *Journal of endodontics* **21**: 277-80.
- Katsikogianni, M. and Y. F. Missirlis (2004). "Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions." *European cells & materials* **8**: 37-57.

- Kho, P. and J. C. Baumgartner (2006). "A comparison of the antimicrobial efficacy of NaOCl/biopure MTAD versus NaOCl/EDTA against *Enterococcus faecalis*." *J Endod* **32**: 652-5.
- Kim, S. and S. Kratchman (2006). "Modern endodontic surgery concepts and practice: a review." *J Endod* **32**: 601-23.
- Kim, S. Y., Y. Shin, C. Y. Lee and I. Y. Jung (2013). "In Vivo Quantitative Evaluation of Live and Dead Bacteria in Root Canal Infection by Using Propidium Monoazide with Real-Time PCR." *Journal of endodontics* **39**: 1359-63.
- Kishen, A., S. George and R. Kumar (2006). "Enterococcus faecalis-mediated biomineralized biofilm formation on root canal dentine in vitro." *J Biomed Mater Res A* **77**: 406-15.
- Kishen, A., S. George and R. Kumar (2006). "Enterococcus faecalis-mediated biomineralized biofilm formation on root canal dentine in vitro." *J Biomed Mater Res A* **77A**: 406-15.
- Kovac, J. and D. Kovac (2011). "Effect of irrigating solutions in endodontic therapy." *Bratislavske lekarske listy* **112**: 410-5.
- Lee, L. W., Y. L. Lee, S. H. Hsiao and H. P. Lin (2016). "Bacteria in the apical root canals of teeth with apical periodontitis." *Journal of the Formosan Medical Association = Taiwan yi zhi*.
- Liu, H., X. Wei, J. Ling, W. Wang and X. Huang (2010). "Biofilm formation capability of *Enterococcus faecalis* cells in starvation phase and its susceptibility to sodium hypochlorite." *J Endod* **36**: 630-5.
- Love, R. M. and H. F. Jenkinson (2002). "Invasion of dentinal tubules by oral bacteria." *Critical reviews in oral biology and medicine* **13**: 171-83.

- Ma, J., Y. Shen, Y. Yang, Y. Gao, P. Wan, Y. Gan, P. Patel, A. Curtis, M. Khakpour and M. Haapasalo (2015). "In vitro study of calcium hydroxide removal from mandibular molar root canals." *Journal of endodontics* **41**: 553-8.
- Ma, J., Y. Shen, Y. Yang, Y. Gao, P. Wan, Y. Gan, P. Patel, A. Curtis, M. Khakpour and M. Haapasalo (2015). "In vitro study of calcium hydroxide removal from mandibular molar root canals." *Journal of endodontics* **41**: 553-8.
- Ma, J. Z., Z. J. Wang, Y. Shen and M. Haapasalo (2011). "A New Noninvasive Model to Study the Effectiveness of Dentin Disinfection by Using Confocal Laser Scanning Microscopy." *J Endod* **37**: 1380-5.
- Mader, C. L., J. C. Baumgartner and D. D. Peters (1984). "Scanning electron microscopic investigation of the smeared layer on root canal walls." *J Endod* **10**: 477-83.
- Malentacca, A., U. Uccioli, F. Mannocci, B. Bhuvu, D. Zangari, C. Pulella and C. Lajolo (2017). "The comparative effectiveness and safety of three activated irrigation techniques in the isthmus area using a transparent tooth model." *Int Endod J*.
- Malentacca, A., U. Uccioli, D. Zangari, C. Lajolo and C. Fabiani (2012). "Efficacy and Safety of Various Active Irrigation Devices When Used with Either Positive or Negative Pressure: An In Vitro Study." *J Endod* **38**: 1622-6.
- Markus Haapasalo, T. U., & Unni Endal (2003). "Persistent, recurrent, and acquired infection of the root canal system post-treatment." *ENDODONTIC TOPICS* **6**: 29-56.
- Markus Haapasalo, U. E., Homan Zandi & Jeffrey M. Coil (2005). "Eradication of endodontic infection by instrumentation and irrigation solutions." *Endodontic Topics* **10**: 77-102.
- Martinho, F. C., L. F. Freitas, G. G. Nascimento, A. M. Fernandes, F. R. M. Leite, A. P. M. Gomes and I. C. G. Camoes (2015). "Endodontic retreatment: clinical comparison of

- reciprocating systems versus rotary system in disinfecting root canals." *Clin Oral Invest* **19**: 1411-7.
- Martins, M., P. Uppuluri, D. P. Thomas, I. A. Cleary, M. Henriques, J. L. Lopez-Ribot and R. Oliveira (2010). "Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms." *Mycopathologia* **169**: 323-31.
- Marton, I. J. and C. Kiss (2000). "Protective and destructive immune reactions in apical periodontitis." *Oral microbiology and immunology* **15**: 139-50.
- Matsumoto, H., Y. Yoshimine and A. Akamine (2011). "Visualization of irrigant flow and cavitation induced by Er:YAG laser within a root canal model." *J Endod* **37**: 839-43.
- McComb, D. and D. C. Smith (1975). "A preliminary scanning electron microscopic study of root canals after endodontic procedures." *J Endod* **1**: 238-42.
- Miller, T. A. and J. C. Baumgartner (2010). "Comparison of the Antimicrobial Efficacy of Irrigation Using the EndoVac to Endodontic Needle Delivery." *J Endod* **36**: 509-11.
- Mohammadi, Z. (2015). "Effects of Root Canal Irrigants on the Planktonic Form of *Enterococcus Faecalis*: A Review." *Nigerian journal of medicine* **24**: 261-7.
- Mohammadi, Z., F. Palazzi, L. Giardino and S. Shalavi (2013). "Microbial biofilms in endodontic infections: an update review." *Biomedical journal* **36**: 59-70.
- Molina, B., G. Glickman, P. Vandrangi and M. Khakpour (2015). "Evaluation of Root Canal Debridement of Human Molars Using the Gentle Wave System." *Journal of endodontics* **41**: 1701-5.
- Moller, A. J., L. Fabricius, G. Dahlen, A. E. Ohman and G. Heyden (1981). "Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys." *Scandinavian journal of dental research* **89**: 475-84.

- Moser, J. B. and M. A. Heuer (1982). "Forces and Efficacy in Endodontic Irrigation Systems." *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **53**: 425-8.
- Nair, P. N., M. Brundin, G. Sundqvist and U. Sjogren (2008). "Building biofilms in vital host tissues: a survival strategy of *Actinomyces radicans*." *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **106**: 595-603.
- Nair, P. N., U. Sjogren, D. Figdor and G. Sundqvist (1999). "Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars." *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **87**: 617-27.
- Nair, P. N., U. Sjogren, G. Krey, K. E. Kahnberg and G. Sundqvist (1990). "Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study." *J Endod* **16**: 580-8.
- Nair, P. N. R., U. Sjogren, D. Figdor and G. Sundqvist (1999). "Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars." *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **87**: 617-27.
- Neelakantan, P., S. Devaraj and N. Jagannathan (2016). "Histologic Assessment of Debridement of the Root Canal Isthmus of Mandibular Molars by Irrigant Activation Techniques Ex Vivo." *J Endod* **42**: 1268-72.
- Nicolas, G. G. and M. C. Lavoie (2011). "[*Streptococcus mutans* and oral streptococci in dental plaque]." *Canadian journal of microbiology* **57**: 1-20.
- Orstavik, D. and M. Haapasalo (1990). "Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules." *Endodontics & dental traumatology* **6**: 142-9.

- Özok AR, Persoon IF, Huse SM, Keijser BJ, Wesselink PR, Crielaard W, Zaura E. Ecology of the microbiome of the infected root canal system: a comparison between apical and coronal root segments. *Int Endod J*. 2012;45:530-41.
- Paque, F., D. K. Rechenberg and M. Zehnder (2012). "Reduction of hard-tissue debris accumulation during rotary root canal instrumentation by etidronic acid in a sodium hypochlorite irrigant." *J Endod* **38**: 692-5.
- Park, E., Y. Shen, M. Khakpour and M. Haapasalo (2013). "Apical Pressure and Extent of Irrigant Flow beyond the Needle Tip during Positive-pressure Irrigation in an In Vitro Root Canal Model." *J Endod* **39**: 511-5.
- Park, E., Y. Shen, M. Khakpour and M. Haapasalo (2013). "Apical pressure and extent of irrigant flow beyond the needle tip during positive-pressure irrigation in an in vitro root canal model." *Journal of endodontics* **39**: 511-5.
- Peciuliene, V., I. Balciuniene, H. M. Eriksen and M. Haapasalo (2000). "Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population." *J Endod* **26**: 593-5.
- Peciuliene, V., A. H. Reynaud, I. Balciuniene and M. Haapasalo (2001). "Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis." *Int Endod J* **34**: 429-34.
- Peeters, H. H. and L. Mooduto (2013). "Radiographic examination of apical extrusion of root canal irrigants during cavitation induced by Er,Cr:YSGG laser irradiation: an in vivo study." *Clinical oral investigations* **17**: 2105-12.
- Peters, O. A., S. Bardsley, J. Fong, G. Pandher and E. Divito (2011). "Disinfection of root canals with photon-initiated photoacoustic streaming." *J Endod* **37**: 1008-12.

- Pinheiro, E. T., B. P. F. A. Gomes, C. C. R. Ferraz, E. L. R. Sousa, F. B. Teixeira and F. J. Souza (2003). "Microorganisms from canals of root-filled teeth with periapical lesions." *Int Endod J* **36**: 1-11.
- Plotino, G., T. Cortese, N. M. Grande, D. P. Leonardi, G. Di Giorgio, L. Testarelli and G. Gambarini (2016). "New Technologies to Improve Root Canal Disinfection." *Brazilian dental journal* **27**: 3-8.
- Portenier, I., T. Waltimo, D. Orstavik and M. Haapasalo (2005). "The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments." *J Endod* **31**: 380-6.
- Ricucci, D. and G. Bergenholtz (2003). "Bacterial status in root-filled teeth exposed to the oral environment by loss of restoration and fracture or caries--a histobacteriological study of treated cases." *International endodontic journal* **36**: 787-802.
- Ricucci, D. and J. F. Siqueira, Jr. (2010). "Fate of the tissue in lateral canals and apical ramifications in response to pathologic conditions and treatment procedures." *J Endod* **36**: 1-15.
- Rocas, I. N., I. Y. Jung, C. Y. Lee and J. F. Siqueira (2004). "Polymerase chain reaction identification of microorganisms in previously root-filled teeth in a South Korean population." *J Endod* **30**: 504-8.
- Rocas, I. N., K. C. Lima, I. V. Assuncao, P. N. Gomes, I. V. Bracks and J. E. Siqueira (2015). "Advanced Caries Microbiota in Teeth with Irreversible Pulpitis." *Journal of endodontics* **41**: 1450-5.



- Rocas, I. N., K. C. Lima, I. V. Assuncao, P. N. Gomes, I. V. Bracks and J. F. Siqueira, Jr. (2015). "Advanced Caries Microbiota in Teeth with Irreversible Pulpitis." *Journal of endodontics* **41**: 1450-5.
- Rocas, I. N. and J. F. Siqueira, Jr. (2012). "Characterization of microbiota of root canal-treated teeth with posttreatment disease." *Journal of clinical microbiology* **50**: 1721-4.
- Rodrigues, R. C., H. S. Antunes, M. A. Neves, J. F. Siqueira, Jr. and I. N. Rocas (2015). "Infection Control in Retreatment Cases: In Vivo Antibacterial Effects of 2 Instrumentation Systems." *Journal of endodontics* **41**: 1600-5.
- Rolph, H. J., A. Lennon, M. P. Riggio, W. P. Saunders, D. MacKenzie, L. Coldero and J. Bagg (2001). "Molecular identification of microorganisms from endodontic infections." *J Clin Microbiol* **39**: 3282-9.
- Russell, A. D. and M. J. Day (1993). "Antibacterial Activity of Chlorhexidine." *J Hosp Infect* **25**: 229-38.
- Sakamoto, M., Y. Takeuchi, M. Umeda, I. Ishikawa and Y. Benno (2001). "Rapid detection and quantification of five periodontopathic bacteria by real-time PCR." *Microbiol Immunol* **45**: 39-44.
- Santos, A. L., J. F. Siqueira, Jr., I. N. Rocas, E. C. Jesus, A. S. Rosado and J. M. Tiedje (2011). "Comparing the bacterial diversity of acute and chronic dental root canal infections." *Plos One* **6**: e28088.
- Santos, A. L., J. F. Siqueira, I. N. Rocas, E. C. Jesus, A. S. Rosado and J. M. Tiedje (2011). "Comparing the Bacterial Diversity of Acute and Chronic Dental Root Canal Infections." *Plos One* **6**.

- Sedgley, C., G. Buck and O. Appelbe (2006). "Prevalence of *Enterococcus faecalis* at Multiple Oral Sites in Endodontic Patients Using Culture and PCR." *J Endod* **32**: 104-9.
- Sen, B. H., P. R. Wesselink and M. Turkun (1995). "The smear layer: a phenomenon in root canal therapy." *Int Endod J* **28**: 141-8.
- Shen, Y., Y. Gao, W. Qian, N. D. Ruse, X. D. Zhou, H. K. Wu and M. Haapasalo (2010). "Three-dimensional Numeric Simulation of Root Canal Irrigant Flow with Different Irrigation Needles." *J Endod* **36**: 884-9.
- Shen, Y., W. Qian, C. Chung, I. Olsen and M. Haapasalo (2009). "Evaluation of the Effect of Two Chlorhexidine Preparations on Biofilm Bacteria In Vitro: A Three-Dimensional Quantitative Analysis." *J Endod* **35**: 981-5.
- Shen, Y., W. Qian, C. Chung, I. Olsen and M. Haapasalo (2009). "Evaluation of the effect of two chlorhexidine preparations on biofilm bacteria in vitro: a three-dimensional quantitative analysis." *J Endod* **35**: 981-5.
- Shen, Y., S. Stojicic and M. Haapasalo (2010). "Bacterial Viability in Starved and Revitalized Biofilms: Comparison of Viability Staining and Direct Culture." *J Endod* **36**: 1820-3.
- Shen, Y., S. Stojicic and M. Haapasalo (2011). "Antimicrobial Efficacy of Chlorhexidine against Bacteria in Biofilms at Different Stages of Development." *J Endod* **37**: 657-61.
- Sigurdsson, A., R. W. Garland, K. T. Le and S. M. Woo (2016). "12-month Healing Rates after Endodontic Therapy Using the Novel GentleWave System: A Prospective Multicenter Clinical Study." *Journal of endodontics* **42**: 1040-8.
- Sigurdsson, A., R. W. Garland, K. T. Le and S. M. Woo (2016). "12-month Healing Rates after Endodontic Therapy Using the Novel GentleWave System: A Prospective Multicenter Clinical Study." *J Endod* **42**: 1040-8.

- Sigurdsson, A., K. T. Le, S. M. Woo, S. A. Rassoulian, K. McLachlan, F. Abbassi and R. W. Garland (2016). "Six-month healing success rates after endodontic treatment using the novel GentleWave System: The pure prospective multi-center clinical study." *Journal of clinical and experimental dentistry* **8**: e290-8.
- Singla, M. G., A. Garg and S. Gupta (2011). "MTAD in endodontics: an update review." *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **112**: e70-6.
- Siqueira, J. F., Jr. and I. N. Rocas (2005). "Exploiting molecular methods to explore endodontic infections: Part 1--current molecular technologies for microbiological diagnosis." *Journal of endodontics* **31**: 411-23.
- Siqueira, J. F., Jr. and I. N. Rocas (2009). "Diversity of endodontic microbiota revisited." *J Dent Res* **88**: 969-81.
- Siqueira, J. F., Jr., I. N. Rocas, A. Favieri and K. C. Lima (2000). "Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite." *J Endod* **26**: 331-4.
- Siqueira, J. F., A. G. Machado, R. M. Silveira, H. P. Lopes and M. DeUzeda (1997). "Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal in vitro." *Int Endod J* **30**: 279-82.
- Siqueira, J. F. and I. N. Rocas (2004). "Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment." *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **97**: 85-94.

Siren, E. K., M. P. P. Haapasalo, K. Ranta, P. Salmi and E. N. J. Kerosuo (1997).

"Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation." *Int Endod J* **30**: 91-5.

Siu, C. and J. C. Baumgartner (2010). "Comparison of the debridement efficacy of the EndoVac irrigation system and conventional needle root canal irrigation in vivo." *J Endod* **36**: 1782-5.

Sjogren, U., D. Figdor, S. Persson and G. Sundqvist (1997). "Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis." *Int Endod J* **30**: 297-306.

Sjogren, U., D. Figdor, S. Persson and G. Sundqvist (1998). "Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis (vol 30, pg 297, 1997)." *Int Endod J* **31**: 148-9.

Skucaite, N., V. Peciuliene, A. Vitkauskiene and V. Machiulskiene (2010). "Susceptibility of endodontic pathogens to antibiotics in patients with symptomatic apical periodontitis." *J Endod* **36**: 1611-6.

Snjaric, D., Z. Carija, A. Braut, A. Halaji, M. Kovacevic and D. Kuis (2012). "Irrigation of human prepared root canal - ex vivo based computational fluid dynamics analysis." *Croat Med J* **53**: 470-9.

Socransky, S. S. and A. D. Haffajee (2002). "Dental biofilms: difficult therapeutic targets." *Periodontology 2000* **28**: 12-55.

Spoleti, P., M. Siragusa and M. J. Spoleti (2003). "Bacteriological evaluation of passive ultrasonic activation." *J Endod* **29**: 12-4.

Stojicic, S., Y. Shen and M. Haapasalo (2013). "Effect of the Source of Biofilm Bacteria, Level of Biofilm Maturation, and Type of Disinfecting Agent on the Susceptibility of Biofilm Bacteria to Antibacterial Agents." *J Endod* **39**: 473-7.

Stojicic, S., S. Zivkovic, W. Qian, H. Zhang and M. Haapasalo (2010). "Tissue dissolution by sodium hypochlorite: effect of concentration, temperature, agitation, and surfactant." *J Endod* **36**: 1558-62.

Stoodley, P., K. Sauer, D. G. Davies and J. W. Costerton (2002). "Biofilms as complex differentiated communities." *Annu Rev Microbiol* **56**: 187-209.

Sundqvist, G. (1992). "Associations between Microbial Species in Dental Root-Canal Infections." *Oral Microbiol Immun* **7**: 257-62.

Sundqvist, G., D. Figdor, S. Persson and U. Sjogren (1998). "Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment." *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **85**: 86-93.

Tambe, V. H., P. S. Nagmode, J. R. Vishwas, S. K. P, P. Angadi and F. M. Ali (2013). "Evaluation of the Amount of Debris extruded apically by using Conv-entional Syringe, Endovac and Ultrasonic Irrigation Technique: An In Vitro Study." *Journal of international oral health* **5**: 63-6.

Tanomaru-Filho, M., L. M. Miano, G. M. Chavez-Andrade, F. F. Torres, T. Leonardo Rde and J. M. Guerreiro-Tanomaru (2015). "Cleaning of Root Canal System by Different Irrigation Methods." *The journal of contemporary dental practice* **16**: 859-63.

Tatikonda, A., N. Sudheep, K. P. Biswas, K. Gowtham, S. Pujari and P. Singh (2017). "Evaluation of Bacteriological Profile in the Apical Root Segment of the Patients with Primary Apical Periodontitis." *The journal of contemporary dental practice* **18**: 44-8.

- Topcuoglu, H. S., A. Akti, S. Duzgun, K. T. Ceyhanli and G. Topcuoglu (2015). "Effectiveness of different irrigation procedures for removal of dentin debris from a simulated internal resorption cavity." *The International journal of artificial organs* **38**: 165-9.
- Townsend, C. and J. Maki (2009). "An In Vitro Comparison of New Irrigation and Agitation Techniques to Ultrasonic Agitation in Removing Bacteria From a Simulated Root Canal." *J Endod* **35**: 1040-3.
- Tran, K. T., M. Torabinejad, S. Shabahang, B. Retamozo, R. M. Aprecio and J. W. Chen (2013). "Comparison of efficacy of pulverization and sterile paper point techniques for sampling root canals." *Journal of endodontics* **39**: 1057-9.
- Tsesis, I., E. Rosen, D. Schwartz-Arad and Z. Fuss (2006). "Retrospective evaluation of surgical endodontic treatment: traditional versus modern technique." *J Endod* **32**: 412-6.
- Turkel, E., E. O. Onay and M. Ungor (2017). "Comparison of Three Final Irrigation Activation Techniques: Effects on Canal Cleanness, Smear Layer Removal, and Dentinal Tubule Penetration of Two Root Canal Sealers." *Photomedicine and laser surgery*.
- van der Sluis, L. W., G. Gambarini, M. K. Wu and P. R. Wesselink (2006). "The influence of volume, type of irrigant and flushing method on removing artificially placed dentine debris from the apical root canal during passive ultrasonic irrigation." *International endodontic journal* **39**: 472-6.
- Vianna, M. E., H. P. Horz, B. P. F. A. Gomes and G. Conrads (2005). "Microarrays complement culture methods for identification of bacteria in endodontic infections." *Oral Microbiol Immun* **20**: 253-8.
- Wade, W., H. Thompson, A. Rybalka and S. Vartoukian (2016). "Uncultured Members of the Oral Microbiome." *Journal of the California Dental Association* **44**: 447-56.

- Wade, W. G. (2013). "The oral microbiome in health and disease." *Pharmacol Res* **69**: 137-43.
- Wade, W. G. (2013). "The oral microbiome in health and disease." *Pharmacol Res* **69**: 137-43.
- Wang, S., Z. Deng, C. J. Seneviratne, G. S. Cheung, L. Jin, B. Zhao and C. Zhang (2015).  
"Enterococcus faecalis promotes osteoclastogenesis and semaphorin 4D expression."  
*Innate immunity* **21**: 726-35.
- Wang, Z. J., Y. Shen and M. Haapasalo (2012). "Effectiveness of Endodontic Disinfecting  
Solutions against Young and Old Enterococcus faecalis Biofilms in Dentin Canals." *J  
Endod* **38**: 1376-9.
- Wang, Z. J., Y. Shen and M. Haapasalo (2014). "Dentin Extends the Antibacterial Effect of  
Endodontic Sealers against Enterococcus faecalis Biofilms." *J Endod* **40**: 505-8.
- Wang, Z. J., Y. Shen, J. Z. Ma and M. Haapasalo (2012). "The Effect of Detergents on the  
Antibacterial Activity of Disinfecting Solutions in Dentin." *J Endod* **38**: 948-53.
- Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas and J. S. Mattick (2002). "Extracellular DNA  
required for bacterial biofilm formation." *Science* **295**: 1487.
- Williamson, A. E., J. W. Cardon and D. R. Drake (2009). "Antimicrobial susceptibility of  
monoculture biofilms of a clinical isolate of Enterococcus faecalis." *J Endod* **35**: 95-7.
- Wilson, M. J., A. J. Weightman and W. G. Wade (1997). "Applications of molecular ecology in  
the characterization of uncultured microorganisms associated with human disease." *Rev  
Med Microbiol* **8**: 91-101.
- Wittgow, W. C., Jr. and C. B. Sabiston, Jr. (1975). "Microorganisms from pulpal chambers of  
intact teeth with necrotic pulps." *J Endod* **1**: 168-71.

- Wohlgemuth, P., D. Cuocolo, P. Vandrangi and A. Sigurdsson (2015). "Effectiveness of the GentleWave System in Removing Separated Instruments." *Journal of endodontics* **41**: 1895-8.
- Wu, M. K. and P. R. Wesselink (2001). "A primary observation on the preparation and obturation of oval canals." *International endodontic journal* **34**: 137-41.
- Yamada, R. S., A. Armas, M. Goldman and P. S. Lin (1983). "A Scanning Electron-Microscopic Comparison of a High Volume Final Flush with Several Irrigating Solutions .3." *J Endod* **9**: 137-42.
- Yang, Y., Y. Shen, Z. J. Wang, X. Y. Huang, H. Maezono, J. Z. Ma, Y. G. Cao and M. Haapasalo (2016). "Evaluation of the Susceptibility of Multispecies Biofilms in Dentinal Tubules to Disinfecting Solutions." *J Endod* **42**: 1246-50.
- Zehnder, M. (2006). "Root canal irrigants." *J Endod* **32**: 389-98.
- Zhu, X., X. Yin, J. W. Chang, Y. Wang, G. S. Cheung and C. Zhang (2013). "Comparison of the antibacterial effect and smear layer removal using photon-initiated photoacoustic streaming aided irrigation versus a conventional irrigation in single-rooted canals: an in vitro study." *Photomedicine and laser surgery* **31**: 371-7.



## Appendices

### Appendix A Calculation of Sample Size (G\*Power)

Sample size was calculated based on the pilot study with G\*Power. The result showed the minimal sample size was 35 when the power was set at 0.95(1-  $\beta$  error problem)

