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Effectiveness of XP-3D Finisher and Irrigants Solutions in Decreasing the Bacterial Load of Intracanal E. faecalis Growth

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A Thesis

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by

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ABSTRACT

Aim & Hypothesis: To assess the efficacy of XP-3D Finisher, in addition to the additive effect of 5.25% NaOCl followed by 2% CHX solutions, in decreasing the load of intracanal *E. faecalis* of infected teeth in vitro. We hypothesize that irrigation XP-3D Finisher, in addition to the additive effect of 5.25% NaOCl followed by irrigation with 2% CHX is superior in disinfecting root canals and reducing *E. faecalis* compared to using 5.25% NaOCl alone.

Materials and Methods: Twenty-four extracted single-rooted teeth with closed apices were infected with *E. faecalis* in vitro for 21 days. Afterward, A glide path with a hand K-type file to the working length was created in all samples. Post contamination bacteriological samples (S1) were collected. Canals were prepared using XP-3D Finisher file and irrigated with 5.25% NaOCl, XP-3D Finisher bacteriological samples (S2) were collected. Canals were irrigated with distilled water, paper dried, and then irrigated with 6 mL of 2% CHX, post CHX irrigation bacteriological samples (S3) were collected. For each of the S1, S2 and S3 samples, the bacterial growth was determined by counting the colony-forming (CFU).

Results: There was a statistically significant difference in the mean bacterial load reduction (CFU) between the three stages of irrigation. Post XP-3D finisher bacteriological samples (S2) showed significant reduction in the number of *E. faecalis* colonies that were able to develop on the agar plates. The XP-3D Finisher sample (S3) completely eliminated *E. faecalis* colonies.

Conclusion: Irrigation with 5.25%, NaOCl in combination with XP-3D Finisher led to a significant reduction in the bacterial load in infected roots. The addition of a final rinse with CHX can further reduce the remaining bacterial load and may improve the overall

antibacterial activity against *E. faecalis* in treated root canals.

DEDICATION

This work is dedicated to my parents, who always believed in me, encouraged me, loved me unconditionally, and always being there whenever I needed them. Thank you from the bottom of my heart. I love you both so much.

To my brother and sister, Sultan and Rajwa. Thank you for your endless support and love. I'm lucky to have you two in my life. To my sister-in-law, Eman. Thank you for your gaudiness and for being a big sister to me.

To my grandparents, uncles and aunties, and my in-laws. Thank you for your words of encouragement and support. Your prayers and blessings are forever appreciated.

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LIST OF ABBREVIATIONS

^{0}C	Celsius
BHI	Brain Heart Infusion
CEJ	Cementoenamel junction
CFUs	Mean colony-forming units
CHX	Chlorhexidine
E. faecalis	Enterococcus faecalis
EDTA	Ethylenediaminetetraacetic acid
kPa	kilopascal
LTA	Lipoteichoic acid
NaOCl	Sodium hypochlorite
Ncm	Torque of 1-newton centimeter
PCA	Para-chloroaniline
PCR	Polymerase chain reaction
PIPS	erbium: yttrium aluminum garnet laser
psi	Pounds per square inch
PUI	Passive ultrasonic irrigation
RPM	Revolutions per minute
SD	Standard deviation

Effectiveness of XP-3D Finisher and Irrigants Solutions in Decreasing the Bacterial Load of Intracanal E. faecalis Growth

I. Introduction

Numerous studies have established the role of microorganisms and their by-products in the pathogenesis of pulpal and periradicular diseases. Endodontic treatment of an infected tooth aims to eliminate the existing infection and prevent microorganisms from infecting or re-infecting the root canal and/or periradicular tissues. [1] In a classical study by Kakehashi et al., germ-free rats did not develop pulpal or periradicular disease following surgical pulp exposure compared to conventional laboratory rats. [2] Möller et al. had comparable results in a monkey study where noninfected necrotic pulp tissue did not cause inflammatory reactions in the apical tissues. Teeth with infected pulp tissue, on the other hand, displayed clinical, radiological, and inflammatory responses. [3] Mechanical root canal debridement can reduce the microbial load inside the canal. However, because of the complexity of the root canal anatomy and the numerous fins and isthmuses, all the instrumentation techniques, when tested, left at least 35% of the canals' surface area uninstrumented. [4] Therefore, in addition to mechanical root canal debridement, chemical agents, such as intra-canal irrigants solutions and medication containing antimicrobial agents, are necessary to achieve successful endodontic treatment. [5] [6]

Root canal infections are polymicrobial in nature. The bacterial microbial composition changes over time, owing to changes in the root canal ecology and inter-species interactions. During the early stages of infection, the pulp is exposed to greater oxygen tension and availability of nutrients from the oral cavity, which allows facultative bacteria to grow in canal space. This results in an overall higher bacterial number and diversity of bacterial types. [7] On the other hand, asaccharolytic species, which are obligate anaerobes

thrive in the more carbohydrate-depleted, yet protein-rich anaerobic environment found in more established infections, most notably in the apical depths of the root canal. [3 7 8] Primary root canal infections are usually dominated by 40–50 species of Gram-negative strictly anaerobic organisms of the genera Fusobacterium, Prevotella, Porphyromonas, Tannerella, and Treponema. [9 10] Persistent/secondary infections tend to have microbial communities with fewer taxa, predominantly Gram-positive facultative anaerobes such Streptococcus, Lactobacillus, Actinomyces, and Enterococcus. [11 12] Enterococcus faecalis (*E. faecalis)* bacteria are significantly linked to persistent/secondary infections. While this species is found in 18% of cases of primary endodontic infections, it was found in 67 % of root-filled teeth. According to odds ratio calculations, failure cases are nearly nine times more likely to have *E. faecalis* than primary infections. [13]

E. faecalis is a Gram-positive, non-spore-forming, fermentative coccus. It is generally identified in the human oral cavity, gastrointestinal system, and female genital tracts. It has shown an excellent adaption to conditions with high nutritional density, low oxygen levels, and a complicated ecology. [14] This bacterium has an oval shape and it ranges between 0.5-1 μm in diameter. *E. faecalis* can exist as single pairs or in small chains colonies in the canal space. Nonhemolytic and nonmotile strains are the most common in a dental infections. When grown on blood or nutrient agar, *E. faecalis* colonies appear round and smooth. Of the strains that are involved in pulpal pathosis, they can grow at 10-45 ^{0}C and can withstand 30 minutes of heating up to 60 ^{0}C.

Various definite and potential virulence factors in *E. faecalis* could play a role in its pathogenesis, including 1) presence of lytic enzymes gelatinase and hyaluronidase, which may be involved in tissue damage, 2) aggregation substance which is involved in binding to

leukocytes and connective extracellular matrix, 3) pheromones which is a small linear peptide involved in conjugative transfer of plasmids and chemoattractant for neutrophils, and 4) lipoteichoic acid which is involved in adhesion to host surfaces and stimulates cytokine production by monocytes. *E. faecalis* is known to be associated with asymptomatic cases of primary endodontic infections, rather than with symptomatic patients. This supports the notion that *E. faecalis* bacteria are not particularly virulent microorganisms. Their emergence as pathogens is more likely to be linked to antimicrobial resistance factors than high virulence. [15]

Several studies show that *E. faecalis* is highly resistant to antibacterial agents used in root canal treatment. [16] [17] This property is thought to be attributed to its ability to invade and remain viable within the dentinal tubule by adhering to the exposed unmineralized collagen in the presence of human serum. Furthermore, developing antibiotic-resistant strains, and forming biofilm colonies more resistant to eradicate than planktonic bacteria. [18] [19] In addition, *E. faecalis* has proton pumps which are critical for its survival, especially in a highly alkaline environment. This mechanism allows the bacteria to effectively pump protons across the cytoplasmic membrane and maintain the cytoplasmic pH. It has also been established that *E. faecalis* has a distinct genetic polymorphism. [20] [21] Moreover, these bacteria can withstand starvation in a minimal metabolic state for a prolonged period, and the starved microorganism can recover when it encounters human serum. [22] The gene regulation relevant for the adaptation of *E. faecalis* to alkaline stress conditions was elucidated in 2015; E. *faecalis* expresses 613 distinct genes at pH 10, with 211 genes being up-regulated and 402 genes being down-regulated. Most of the over-regulated genes were related to amino acids and nucleotide transport and metabolism, implying that *E. faecalis* can use some amino acids

as carbon and energy sources in high alkaline conditions to promote pyrimidine production, which can lead to an increase in bacterial virulence. Genes involved in the metabolism of carbohydrates and amino acids, on the other hand, were downregulated. [23]

Sodium hypochlorite (NaOCl) is the most widely used chemical irrigation solution in root canal treatment since Coolidge advocated its usage in endodontic practice in 1919. [24] Commercial sodium hypochlorite solutions are highly alkaline and hypertonic, with nominal chlorine contents ranging from 10 to 14 %. In endodontic practice, NaOCl is utilized as an unbuffered solution at pH 11 and as a bicarbonate-buffering solution at pH 9.0. Studies demonstrate the superior ability of this solution in dissolving organic tissues. When hypochlorous acid releases chlorine, that leads to amino acid degradation and hydrolysis. [25] In addition, NaOCl is effective in eradicating most organisms related to pulp and periradicular infection due to its capability to inhibit bacterial enzymes and cellular metabolism secondary to chlorine oxidation. [26] Moreover, its high pH can cause phospholipid degradation and protein denaturation. [27]

Chlorhexidine (CHX) is another common irrigants solution used in root canal treatment. CHX is a synthetic cationic bis-guanide molecule composed of two symmetric 4-chlorophenyl rings and two biguanide groups linked by a hexamethylene chain. CHX is a positively charged hydrophobic and lipophilic molecule. [28] CHX antibacterial effect is due to the attachment of the cationic molecule to extra-microbial complexes and negatively charged microbial cell walls, which leads to disruption in the osmotic balance of bacteria. Low molecular weight chemicals, such as potassium and phosphorus, are released from CHX at low concentrations, resulting in a bacteriostatic effect. At higher doses, CHX has a

bactericidal effect. This results from protein cross-linking and precipitation and/or coagulation of the cytoplasm of bacterial cells, which leads to their death. [29]

Dentin treated with CHX gains antibacterial substantivity due to the positively charged ions generated by CHX adsorbed into the dentin and prevents microbial colonization on its surface for some time after the medicament has been applied. [30] In different studies, it was discovered that using CHX can suppress microbial activity for 72 hours, seven days, 21 days, four weeks, and up to 12 weeks. [31] [32] [33] [34] [35] [36] Furthermore, 2% chlorhexidine was significantly more effective in decreasing the load of intracanal E. faecalis than the 5.25% NaOCl in both in-vitro and in-vivo laboratory studies. [37] [38] In contrast to NaOCl, CHX cannot dissolve organic tissue. Still, it can be used as an alternative to NaOCl in situations of open apex, root resorption, foramen enlargement, and root perforation, as well as in patients allergic to bleaching solutions due to its biocompatible nature. [39] [40]

The XP-3D Finisher (Brasseler USA®, Savannah, Georgia) has been introduced as an adjunctive approach to improving the effectiveness of instrumentation and irrigation in endodontics (Figure 1). According to the manufacturer, it is made from MaxWire alloy (Martensite-Austenite Electropolish FleX). The instrument is in the martensite phase at room temperature and appears straight. At body temperature, it transitions into an austenite phase. This allows the material to form into a spoon shape, enabling the instrument to expand and contract in response to the shape of the root canal wall. This shape change allows the instrument to debride the root canal system in a 3-dimensional manner and enhance the irrigation process. [41] Using an infected tooth model with an artificial apical groove, the XP-3D Finisher showed superior biofilm removal efficacy inside and outside the canal groove, compared to conventional needle irrigation and passive ultrasonic irrigation (PUI). [42] Using a

confocal laser scanning microscope, it was shown that XP-3D Finisher was more effective in disinfecting the main canal space in the coronal, middle, and apical third and up to 50 microns into tubules than standard needle irrigation, sonically agitating with EndoActivator, and irrigation with erbium: yttrium aluminum garnet laser (PIPS). [43] In another study that assessed the efficacy of the XP-3D Finisher vs. the Endo Activator on debris and smear layer removal in curved canals, the XP-3D Finisher showed similar effectiveness for debris and smear layer removal to Endo Activator. [44] Moreover, in a study evaluating the effectiveness of XP-3D Finisher as an adjunctive approach in *E. faecalis* canal load reduction using polymerase chain reaction (PCR), the authors concluded that both XP-Endo Finisher and passive ultrasonic irrigation exhibited antibacterial effectiveness. Yet, only the XP-Endo Finisher caused a significant reduction in the bacterial counts after chemomechanical preparation. [45]

II. Research Aim and Hypothesis

Research Aim

To assess the efficacy of XP-3D Finisher, in addition to the additive effect of 5.25% NaOCl followed by 2% CHX solutions, in decreasing the load of intracanal *E. faecalis* of infected teeth in vitro.

Hypothesis

We hypothesize that irrigation with XP-3D Finisher, in addition to the additive effect of 5.25% NaOCl followed by irrigation with 2% CHX is superior in disinfecting root canals and reducing *E. faecalis* compared to using XP-3D Finisher and 5.25% NaOCl alone.

II. Significance

The individual disinfecting properties of 5.25% NaOCl solution, 2% CHX solution, and the XP-3D Finisher, have been previously assessed. However, the usage of the XP-3D Finisher in combination with 5.25% NaOCl and thereafter 2% CHX has not been assessed. The purpose of this in-vitro study is to evaluate the disinfecting capacity of the XP-3D Finisher as an adjunctive approach with the combined effect of 5.25% NaOCl and 2% CHX against intracanal *E. faecalis* by determining the mean colony-forming units (CFUs) of remaining bacterial colonies after exposure. This combination may have an additive impact in eliminating *E. faecalis* from the treated root canals, thus potentially increasing a positive treatment outcome and preventing future re-infection.

IV. Materials and Methods

A. Collection of teeth samples

This is an in-vitro study using extracted single-rooted teeth with closed apices. The teeth were collected from the teeth jar in the Gavel Lab, at Tufts University School of Dental Medicine.

Inclusion criteria for the selected teeth were as follows: Oval-shaped teeth based on buccolingual and mesiodistal radiographic projections. To fulfill oval-shaped criteria, The maximal initial horizontal dimension (MaxIWW) must have been at least two times greater than the minimal initial horizontal dimension (MinIWW), at 5 mm short of working length. [46]

Exclusion criteria for selected teeth were as follows: Presence of stains, cracks, hypo-mineralization, hypoplasia, white spot lesions, dental caries, and restorations.

There was no patient identification of any of the included teeth. Once selected to be included in this study, teeth were immersed in 50 mL of 10% NaOCl solution for at least five days according to the Institutional Biosafety Committee (IBC) disinfection approved Gavel Lab recommendations.

B. Preparation of teeth samples

The crowns of teeth were separated from their roots at the cementoenamel junction (CEJ) using a high-speed straight fissure carbide bur (#169) under water cooling. Thereafter, teeth were flushed with air and water for 10 seconds to remove any organic debris. The length of roots was standardized to 10 mm by reduction at the CEJ site. The entire length of each root canal was irrigated for 4 minutes with 2 mL of 5.25% NaOCl followed by neutralization with 2 mL of 3.86% sodium thiosulfate and 2 mL of 17% Ethylenediaminetetraacetic acid (EDTA, pH 7.2) for 4 minutes to remove any organic debris which might have been left together with the smear layer. To ensure complete smear layer removal, roots were placed for 4 minutes in an ultrasonic bath in 5 mL of 5.25% NaOCl solution, neutralized with 5 mL of 3.86% sodium thiosulfate, and then immersed for 4 minutes in 5 mL of 17% EDTA. The attached soft tissue and calculus were removed from the roots with periodontal scalers, and the root surfaces were pumiced clean. Thereafter, the roots were immersed in sterile distilled water in a plastic bag and exposed to pressure in a pressure pot at 103 kPa (15 psi) and 121ºC for 20 minutes to remove any residual irrigants from the dentinal tubules (Figure 2). Buccolingual and mesiodistal radiographic images of the roots were taken to verify the internal canal anatomy. Teeth that were determined not oval were excluded at this point.

The teeth included in this study were placed in distilled water in individual tubes (two roots per tube) and sterilized in an autoclave for 30 min at 121^0 C (Tuttnauer Brinkmann 3545E).

C. In vitro infection of teeth samples

The roots were infected with *E. faecalis* (ATCC 29212 Mini) in vitro, in a procedure that was modified from the procedure described by Haapasalo and Ørstavik. [47] A Frozen mini

cryovial of *E. faecalis* was thawed upright and gently agitated in a 25 ^{0}C-30 ^{0}C water bath for approximately 2-3 minutes until all the ice crystals were melted. Immediately after thawing, the cryovial was wiped with 70 % ethanol and its content was transferred into a 15 mL conical tube that contained 5 mL of Brain Heart Infusion (BHI) broth (BD 237500). The BHI tube was incubated at 37 ^{0}C for 24 hours (Thelco Incubator, Model 4, Precision Scientific). Samples of BHI broth with E. *faecalis* colonies were streaked on BHI agar plates (BD 211065), and the plates were incubated at 37 ^{0}C for 24 hours (Figure 3). Next, individual *E. faecalis* colonies were suspended in 5 mL BHI broth and incubated overnight at 37 ^{0}C. A serial dilution of bacterial suspension was performed. A spectrophotometer was used to determine the optical density for each dilution, to obtain a working concentration of 108 CFU/mL. The corresponding optical density (O.D. at 600 nm, Amersham Pharmacia Biotech, Ultrospec 2000) for each dilution was measured. Our working concentration was 108 CFU/mL, and it has been determined that an optical density of 0.2-0.3 corresponds to a bacterial concentration of 108 CFU/mL based on Haapasalo and Ørstavik's work. [47]

To thoroughly contaminate the canal, the prepared teeth samples were submerged in *E. faecalis* bacterial suspension in BHI broth and incubated at 37 ^{0}C for 21 days. [16] (Figure 4) To avoid medium saturation, 25 mL of the bacterial suspension was be replaced daily with freshly prepared BHI broth. Using a spectrophotometer, the concentration of the bacterial suspension was determined every other day to maintain the desired concentration of 108 CFU/mL. Following 21 days of incubation of the teeth samples with *E. faecalis*, the roots (including the canals) were flushed with 2 mL sterile distilled water, dried with paper points, and their apical foramina obliterated with sterile sticky wax.

D. Experimental Procedures

A glide path was created in all samples using sizes #15, #20, #25, and #30 hand K-type file to the working length. During glide path procedures, the teeth were irrigated with 4 mL 5.25% NaOCl solution.

To obtain dentin samples from the canal lumen, the canal content was agitated with a sterile #20 Hedstrom file in a standardized circumferential filing motion inserted to the working length to obtain dentin samples from the lumen. Next, the file was withdrawn, and its blade was cut off and dropped into an Eppendorf tube (AM9624) pre-filled with 400 µL Phosphate buffered saline (PBS). Subsequently, a sterile paper point (Dentsply-Maillefer, Ballaigues, Switzerland) was introduced into the entire working length of the canal and retained in position for 1 minute. Two additional paper points were introduced into the canal. All paper points were retrieved and added to the blade tip in the Eppendorf tube. (Figure 5).

The bacterial growth was determined by counting the colony-forming units (CFU) based on the method described by Marinho et al. [48] The Eppendorf tubes containing the root canal content were shaken for 1 minute using Vortex Genie 2 (Fisher Scientific 12812). A ten-fold serial dilution was made up to the 10^{-6} by taking 40 µL from the initial concentration. 200 µL of 10^{-0} and 10^{-5} were plated onto BHI agar plates. Once (S1) CFU samples were obtained, the canals were irrigated with 2 mL of sterile distilled water, dried with sterilized paper points, and stored in PBS overnight. Post contamination bacteriological samples (S1) CFU were calculated after 24 hours (Figure 6).

An XP-3D Finisher file was placed 1 mm short of the working length. According to the manufacturer's recommendations, it was operated using an electric torque-controlled motor EndoSync Plus (Brasseler USA®, Savannah, Georgia 5027135U0) at 1000 Revolutions per minute (RPM) and torque of 1-newton centimeter (Ncm) in a vertical motion for 1 minute.

Canals were irrigated with 6mL of 5.25% NaOCl solution during instrumentation. For the final rinse, the canals were irrigated with 1 mL 5.25% NaOCl solution for 1 min. Then, with the same procedure described above for collecting post contamination bacteriological samples (S1), the post XP-3D Finisher bacteriological samples (S2) CFU were calculated (Figure 7).

The canals were irrigated with 6 mL of distilled water, paper dried, and then irrigated with 6 mL of 2% CHX solution. Thereafter, with the same procedure described above for collecting bacteriological samples, the post CHX irrigation bacteriological samples (S3) CFU were calculated (Figure 8).

At the completion of the study and for infection control purposes, all teeth samples, collection tubes, broth tubes, and agar plates were sterilized in an autoclave before they were discarded.

E. Sample size calculation and statistical analysis

A sample size calculation was conducted in nQuery Advisor. To have 80% power, a sample size of 10 was necessary assuming a type I error of 5 %, a mean (SD) of 0.0305 (0.0027) for the sodium hypochlorite treatment, and a mean (SD) of 0.0131 (0.0173) for the chlorhexidine treatment. [49]

Descriptive statistics (means and standard deviations, medians, and interquartile ranges) were calculated. The normality of the data was assessed graphically and with the Shapiro-Wilk test. The assumption of normality was unmet as the data did not follow a normal distribution, and the p-value for the Shapiro-Wilk test was less than 0.05. Differences in percent of bacterial reduction were analyzed between the three stages of irrigation with Friedman's test with the Wilcoxon signed-rank test with the Bonferroni correction for

posthoc pairwise comparisons (p-value cutoff = 0.017). SPSS version 27 was used for the analysis.

V. Results

The culture technique indicated that bacterial contamination was present in 95.83% of the root canals investigated (23/24 teeth). Table 1 provides an overview of descriptive statistics for the treatment groups: for the post contamination bacteriological samples (S1), the mean CFU count was 6.16 *E. faecalis* colonies with a standard deviation of ±7.56. After root canal instrumentation with XP-3D Finisher and irrigation with 5.25% NaOCl (S2), the mean CFU count was reduced to 2.46 *E. faecalis* colonies with a standard deviation of ±2.10. When the post CHX irrigation bacteriological samples (S3) were taken, all the samples had a 100% reduction in the *E. faecalis* colonies. The mean CFU count was 0.00 with a standard deviation of 0.00. When examining each sample for the individual bacterial load reduction (CFU) at different stages of irrigations, the percent reduction from S1 to S2 ranged between 25%-100%. However, in 3 samples of the 24 samples in S2, there was an increase in bacterial load by 1-2 colonies. (Table 2) (Figure 9).

Post-hoc pairwise comparisons with the Wilcoxon signed rank test, and Bonferroni correction demonstrated a statistically significant difference in the mean bacterial load reduction (CFU) between the three stages of irrigations ($p < 0.001$). There was a statistically significant difference between post bacteriological contamination samples (S1) and both the post-XP-3D finisher bacteriological samples (S2) and post CHX irrigation bacteriological

samples (S3) ($p < 0.001$). Moreover, there was a statistically significant difference between post-XP-3D finisher bacteriological samples (S2) and post CHX irrigation bacteriological samples (S3) ($p < 0.001$). (Figure10)

Table 3 provides the percent reduction of bacterial load (CFU) at each time point. Analyzing the post XP-3D finisher bacteriological samples (S2), the bacterial load was reduced by 60% (59/148 colonies) and by 100% (148/148 colonies) post XP-3D -3D Finisher sample (S3).

VI. Discussion

This in-vitro study evaluated the application of XP-3D Finisher with an additive effect of 5.25% NaOCl followed by 2% CHX in decreasing the load of intracanal *E. faecalis* using mean colony-forming units (CFUs). Mature *E. faecalis* was selected for this investigation because it is the most common and tenacious bacterium isolated from root canals of previously root-filled teeth with chronic apical periodontitis. Based on a previous study conducted by Ørstavik & Haapasalo, a mature 21-day *E. faecalis* had the potential to infiltrate dental tubules up to 300-400 μm in a bovine model in only one day. At a 21-day contamination period, it has penetrated up to 800 μm, making the canal densely contaminated. [16] Moreover, mature bacteria were more resistant to root canal irrigants. It was hypothesized that in young biofilms, more bacteria are in the active, exponential growth phase, and the biofilms' structural development, such as the synthesis of the extracellular polymeric matrix, has not yet been completed. Consequently, they are less resistant than mature bacterial biofilm. [50]

Oval-shaped canals were tested in this study since they are quite challenging root canal configurations to clinicians[51] [52] [53] They are hard to clean, shape, and fill, which may lead to an increase in the bacterial load in the canal and, consequently, reduction in the prognosis of root canal treatment. A sampling of bacteria from root canals can be done using

paper points. However, paper points absorb only the fluid in the canal lumen, disregarding bacteria in the dentinal tubules. [54] Therefore, in this study to reduce the potential of false-negative culture results, we used both paper points and standardized circumferential filing motion to the working length with the #20 Hedstrom file to ensure obtaining dentin samples from the canal. In three out of the 24 teeth samples, we found an increase of 1-2 bacterial colonies after using XP-3D Finisher combined with 5.25% NaOCl (S2). A potential explanation for this is that genetically identical bacteria might behave very differently. Researchers from the University of Washington discovered that when a bacterial cell divides into two daughter cells, the distribution of cellular organelles might be uneven. Depending on whatever parts of the split they received, the resultant cells may behave differently. Their discovery could explain how bacteria survive antibacterial treatments by maintaining a slow-growing, resting condition in some of their populations. Antibacterial treatments target fast-growing cells; thus, these dormant cells have a better chance of surviving the treatment. [55]

This research has demonstrated that when using XP-3D Finisher in combination with 5.25% NaOCl against *E. faecalis*, the bacterial load was significantly reduced by 60%. Yet, it is unclear if this reduction was due to the impact of 5.25% NaOCl irrigation alone or the additive effect of the XP-3D Finisher on the activity of this solution. In previously published studies, XP-3D Finisher was compared to PUI as an adjunctive step for disinfection of the mandibular molar root canal system. XP-3D Finisher alone led to a reduction in *E. faecalis* bacterial load (as measured by CFU) in the main canal by 70%, in comparison to the 50% reduction that was achieved by PUI. However, neither XP-Endo nor PUI successfully cleaned the isthmus/recess zones of mandibular molars. [45] A comparable result in a study by Carvalho *et al.*, single-file instrumentation using XP-3D Shaper and Reciproc Blue files

successfully lowered bacterial loads in oval-shaped root canals. Adding the XP-3D Finisher to the irrigation/instrumentation procedure increased the cleaning efficiency of both file systems but did not entirely eradicate the bacterial contamination. [56] Literature shows varied results regarding the effectiveness of both NaOCl and 2 % CHX solutions against *E. faecalis*. This research study demonstrated complete bacterial reduction when a final 2 % CHX rinse was added to the irrigation protocol. In agreement with our findings, a study tested the antibacterial effects of 5.25% NaOCl and 2% CHX against *E. faecalis* after 5 minutes and after 48 hours. At both periods, the 2 % CHX was much more effective against *E. faecalis* than the 5.25 % NaOCl.[37] Zamany *et al.* also investigated the effects of supplementing the standard treatment regimen with a 2% CHX final rinse. Their findings revealed that one of the CHX cases had cultivable bacteria, but seven of the 12 control cases who were irrigated with 1% NaOCl had cultivable bacteria; this difference was statistically significant. [57] In another study, when the antibacterial solution against both 1-day-old and 3-week-old biofilms was investigated, the most effective antibacterial solution was 6% NaOCl. The quickest reduction in the survival of bacteria in *E. faecalis* biofilms occurred in the first 3 minutes, then slowed dramatically after 10 minutes. There was no statistically significant difference in bacterial death between 2 % chlorhexidine and 2 % NaOCl. [50] Another study showed that 2.5% NaOCl and 0.12% CHX had comparable antibacterial properties and concluded that both solutions could be used as irrigants. [58] Variances in experimental methods, concentration, irrigating solution, patient and anatomical differences in root canal structure or the duration of time utilized in the analysis are likely to contribute to these differences.

Since CHX has significant antibacterial action, it has been used as a critical component of irrigating solutions and intracanal medicaments during root canal therapy. CHX has been shown to have greater antibacterial efficacy against Gram-positive bacteria than against Gram-negative bacteria[59] [60] CHX is a cationic bisguanide that appeared to cause intracellular leakage by adhering to the phosphate groups of the bacterial cell wall, which are negatively charged. [28] As Gram-positive bacteria, *E. faecalis* is more sensitive to cations since the cell walls of these microorganisms are negatively charged. Moreover, the greater antibacterial efficacy of CHX on Gram-positive bacteria could also be due to differences in cell wall features of Gram-positive and Gram negative bacteria. Gram-negative bacteria have a more complex cell wall that is less permeable and susceptible to CHX than Gram-positive bacteria.[61] According to a recent study, the Lipoteichoic acid (LTA), a major immuno-stimulatory component of the *E. faecalis* cell wall, can be inactivated by CHX treatment, which may explain the high effectivity of this solution against *E. faecalis*. [62]

Although Irrigation with NaOCl followed by a final rinse with CHX may improve overall antibacterial activity, chemical interactions between the irrigants should also be considered. When NaOCl and CHX are mixed, some studies have documented color change and precipitation containing mostly para-chloroaniline (PCA). [63] [64] When CHX is combined with NaOCl, the CHX molecules are hydrolyzed into smaller fragments, each of which becomes a by-product. Because of the low bond dissociation energy between the two atoms, it is speculated that the bond between carbon and nitrogen is the first bond to be broken in this reaction. Molecules that have low bond dissociation energies are more likely to break. PCA, among other fragments, is formed because of this disassociation. It has been shown that as the concentration of NaOCl increased, the amount of PCA increased, as well. PCA

has been demonstrated to be hazardous, toxic, and potentially carcinogenic. [65] [37] Furthermore, there are concerns that the precipitate may interfere with the root filling sealability. [66] Therefore, before the final CHX rinse, the canal must be dried with paper points and irrigated with distilled water. [63]

This study had some limitations. First, it was an in-vitro study, therefore the precise intraoral conditions were not replicated. Secondly, it was not possible to conclude if the reduction in CFU of *E. faecalis* in the second sample (S2) was due to the additive effect of the XP-3D Finisher or the sole impact of the 5.25% NaOCl irrigation due to the lack of control groups to compare the results to. Lastly, irrigation of the root canal with 5.25% NaOCL while creating a glide path may influence the bacterial reduction seen on the second sample due to the increased time of the NaOCL exposure.

Future studies may help to define the individual effect of XP-3D Finisher on the reduction of bacterial loads in infected root canals. Also, various irrigation protocols can be tested using a polymicrobial model, as root canal infections are polymicrobial in nature.

VII. Conclusion

Within the limitations of this in vitro study, it was concluded that irrigation with 5.25%, NaOCl, in combination with XP-3D Finisher filing, led to a significant reduction in the bacterial load. Adding a final rinse with 2% CHX further reduced the bacterial load and improved the overall antibacterial activity of these solutions against *E. faecalis.* As a result, this study emphasizes the importance of adding 2% CHX as a final rinse in the irrigation protocol, especially in retreatment cases. As irrigation with 5.25% NaOCl in combination with adjunctive approach (XP-3D Finisher) led only to a 60 % reduction in E. Faecalis intracranial bacterial load.

Appendix A

Table 1. Descriptive statistics for the treatment groups. N =24

	Mean	Standard deviations	Medians	Interquartile ranges
S1 Bacterial Load (CFU)	6.17	7.56	4.00[A]	4.00
S2 Bacterial Load (CFU)	2.46	2.11	2.00[B]	2.00
S3 Bacterial Load (CFU)	0.00	0.00	0.00[C]	0.00

*Medians which share a letter are not statistically different at the 1.7% level

Table 2. Bacterial load reduction for each sample at different stages of irrigation and differences in the percent of the bacterial reduction.

Sample	S1 Bacterial Load	S2 Bacterial Load	S3 Bacterial Load	S1-S2	S2-S3
1	4	2	0	50%	100%
2	32	8	0	75%	100%
3	6	2	0	67%	100%
4	6	2	0	67%	100%
5	25	1	0	96%	100%
6	6	2	0	67%	100%
7	11	7	0	36%	100%
8	10	4	0	60%	100%
9	10	3	0	70%	100%
10	6	3	0	50%	100%
11	4	6	0	-50%	100%
12	4	3	0	25%	100%
13	4	3	0	25%	100%
14	1	2	0	-100%	100%
15	2	2	0	0%	100%
16	1	1	0	0%	100%
17	2	3	0	-50%	100%
18	3	1	0	67%	100%
19	2	0	0	100%	.

20	2	1	0	50%	100%
21	0	0	0	.	.
22	4	3	0	25%	100%
23	1	0	0	100%	.
24	2	0	0	100%	.

Table 3. Total number of *E. faecalis* colonies and the percent reduction of bacterial load (CFU) at different stages of irrigation

	S1	**S2**	**S3**
Total number of *E. faecalis* Colonies	148	59	0
Percent Reduction of bacterial load *(CFU)*	-	60%	100%

Appendix B

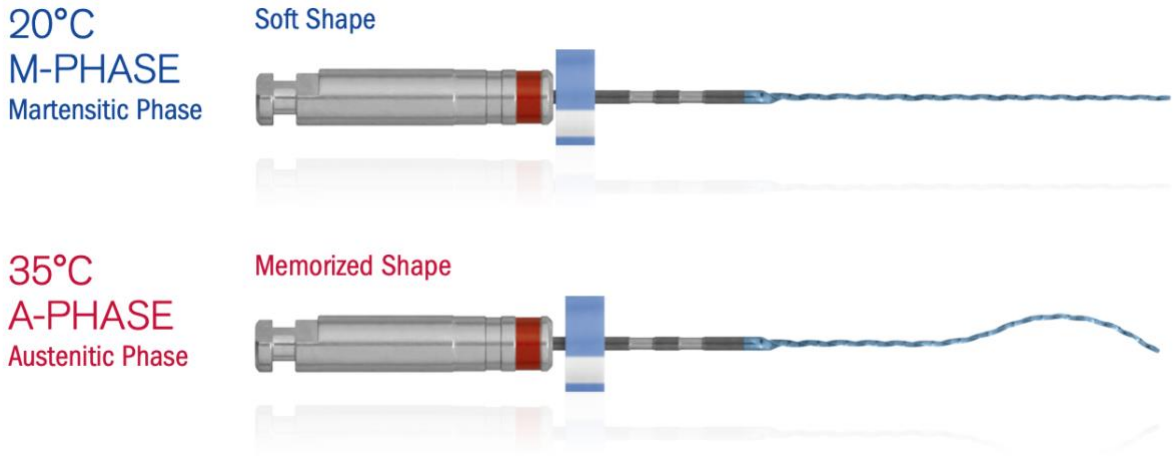

Figure 1. XP-3D Finisher file (Brasseler USA®, Savannah, Georgia)

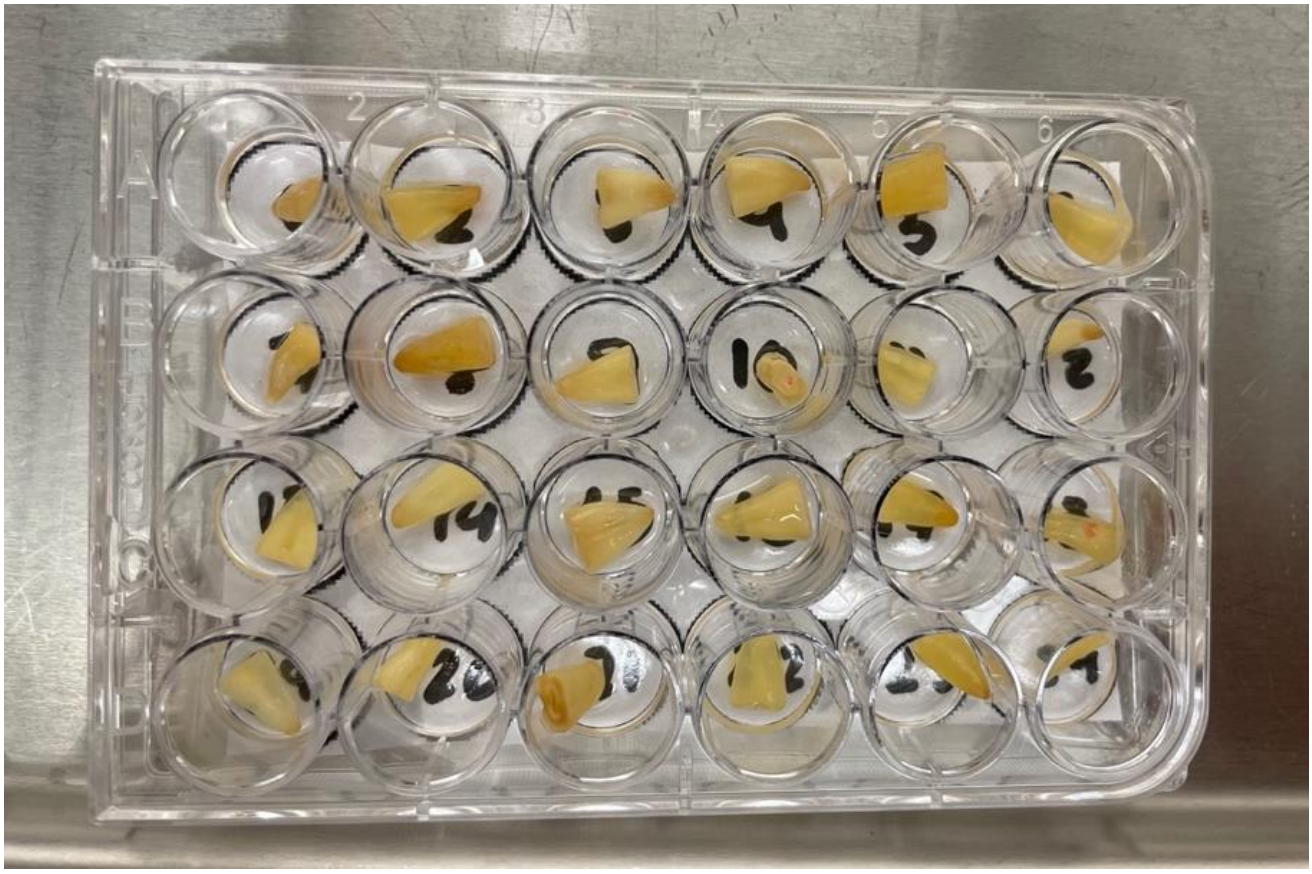

Figure 2. Preparation of teeth samples

Figure 3. Samples of BHI broth with E. *faecalis* colonies were streaked on BHI agar plates (BD 211065)

Figure 4. Sample teeth immersed in bacterial suspension

Figure 5. Samples collected in Phosphate buffered saline (PBS) Eppendorf tube (#20 Hedstrom file and three paper points)

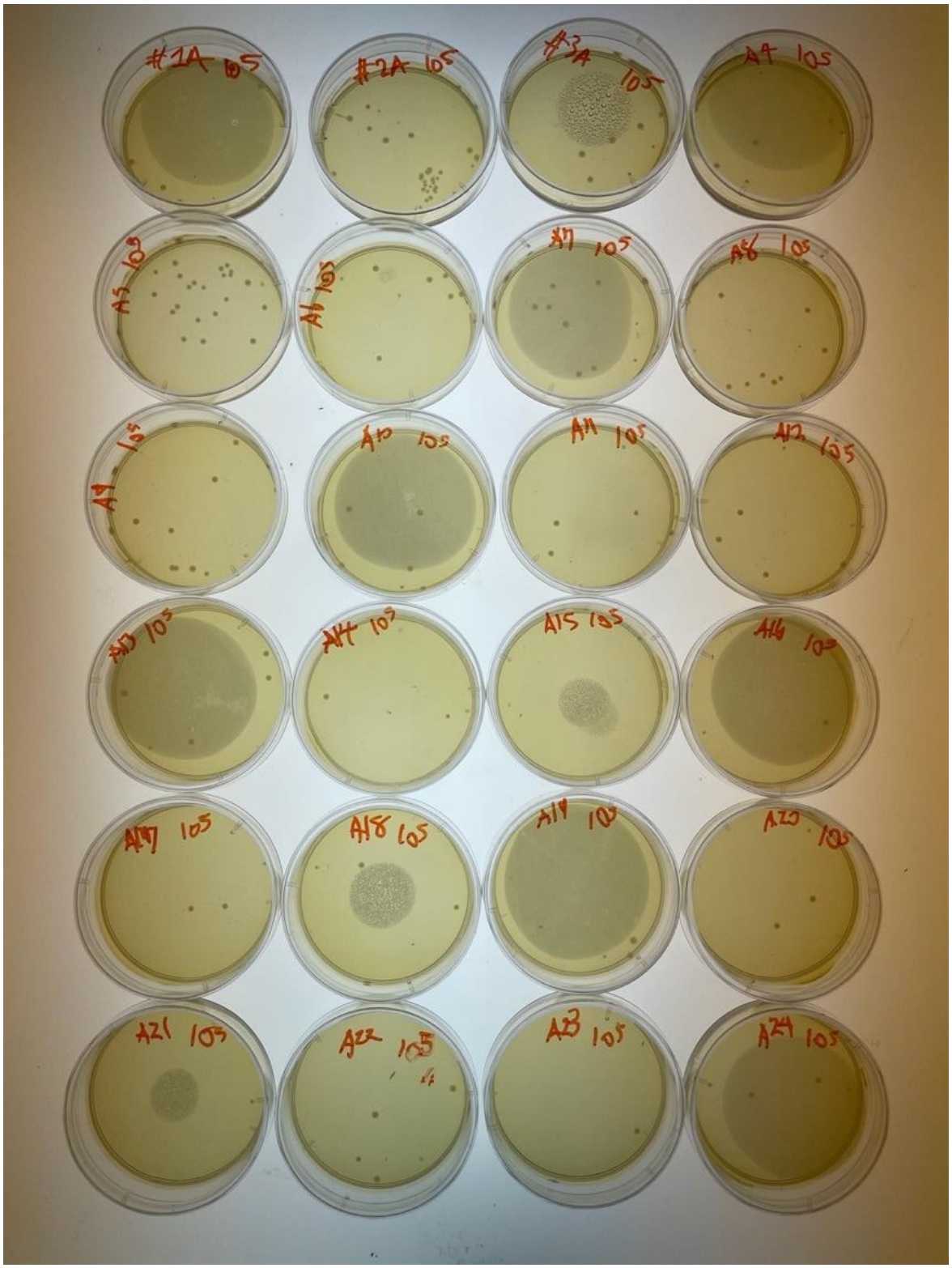

Figure 6. Post contamination bacteriological samples (S1) colony-forming unit (CFU)

Figure 7. Post XP finisher bacteriological samples (S2) colony-forming unit (CFU)

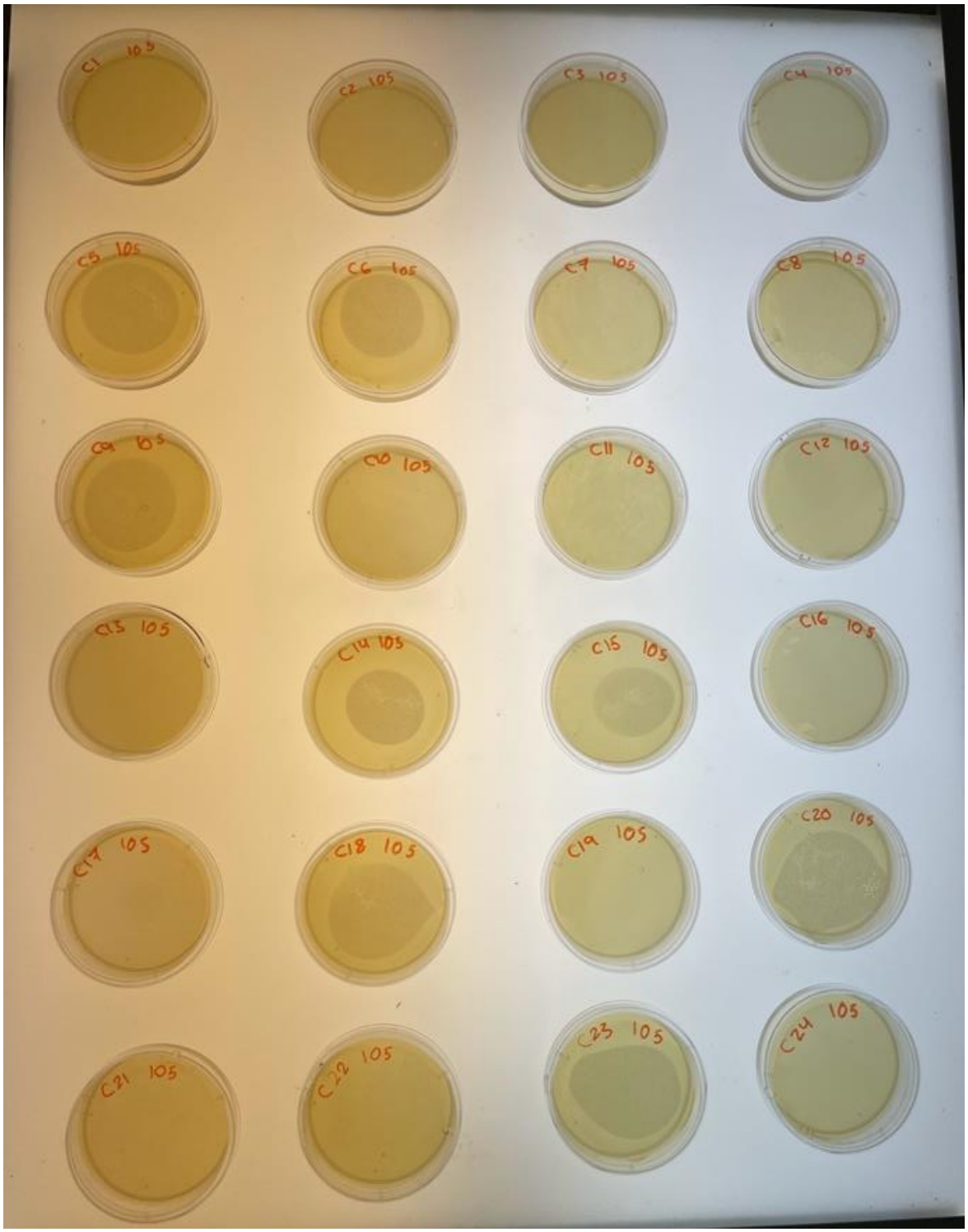

Figure 8. Post irrigation bacteriological samples (S3) colony-forming unit (CFU)

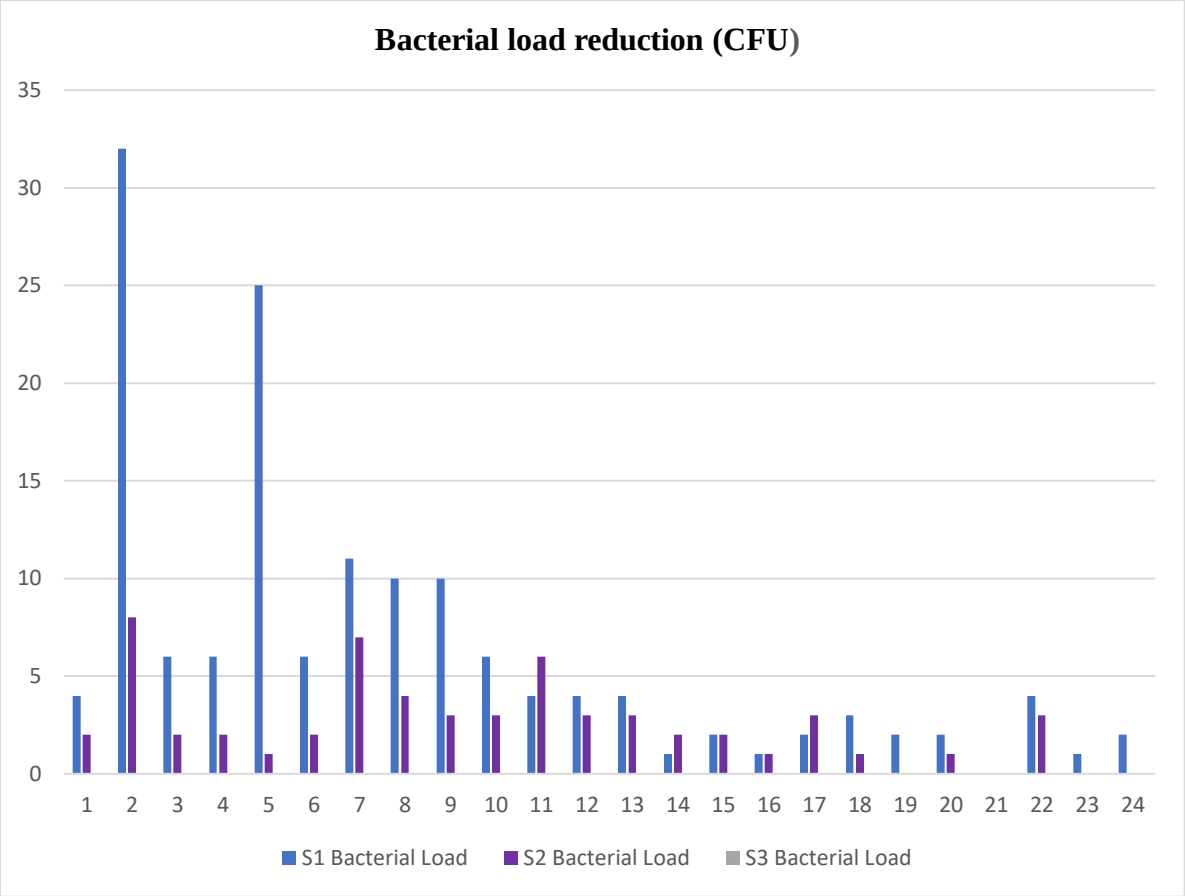

Figure 9. Bacterial load reduction for each sample at different stages of irrigation

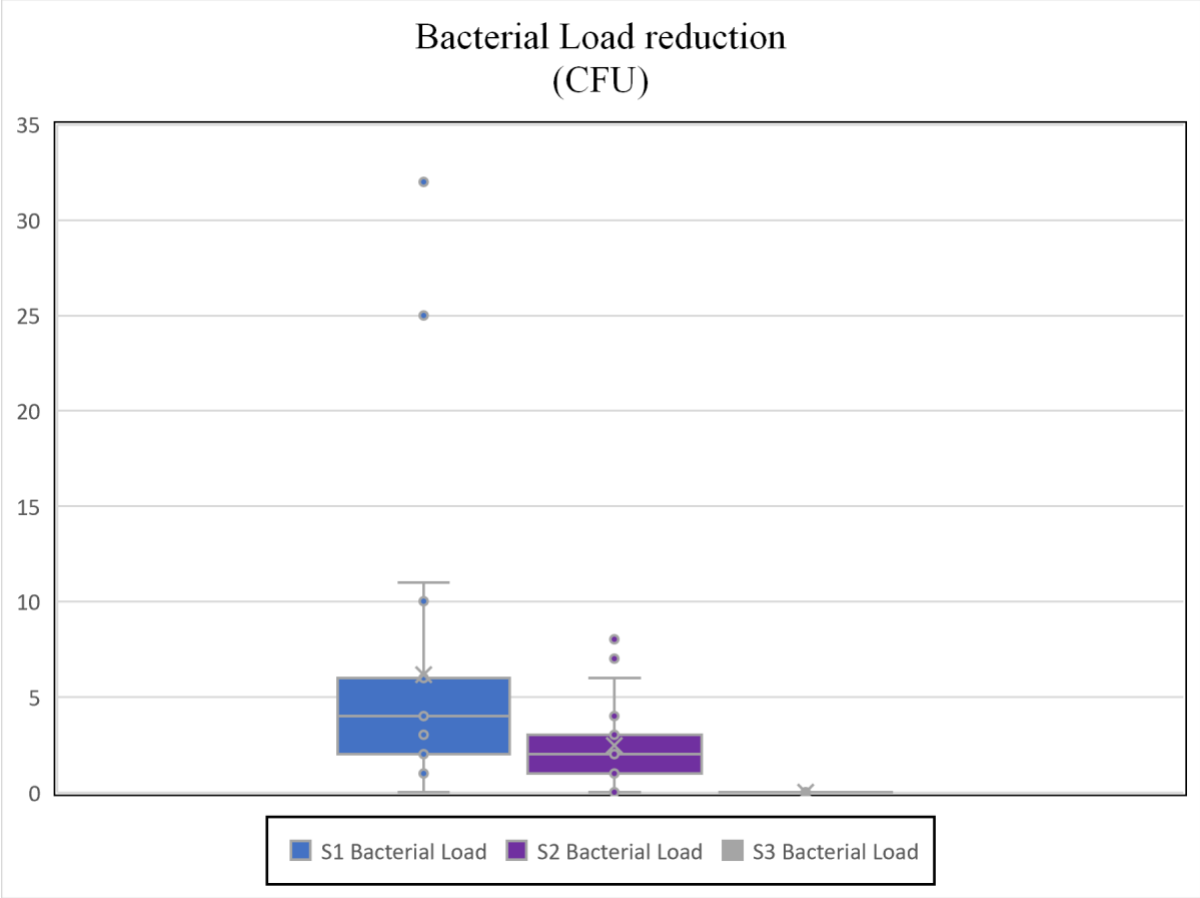

Figure 10. Bacterial load reduction at different stages of irrigation

BIBLIOGRAPHY

1. Narayanan LL, Vaishnavi C. Endodontic microbiology. *J Conserv Dent*. 2010;13(4):233-239. doi:10.4103/0972-0707.73386

2. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surgery, Oral Med Oral Pathol*. 1965;20(3):340-349. doi:10.1016/0030-4220(65)90166-0

3. MÖLLER ÅJR, FABRICIUS L, DAHLÉN G, ÖHMAN AE, HEYDEN G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Eur J Oral Sci*. 1981;89(6):475-484. doi:10.1111/j.1600-0722.1981.tb01711.x

4. Peters, Schönenberger, K., & Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. *Int Endod J*. 2001;34(3):221-230.

5. Haapasalo M, Endal U, Zandi H, Coil JM. Eradication of endodontic infection by instrumentation and irrigation solutions. *Endod Top*. 2005;10(1):77-102. doi:10.1111/j.1601-1546.2005.00135.x

6. Estrela C, Sydney GB, Figueiredo JAP, Estrela CRDA. Antibacterial efficacy of intracanal medicaments on bacterial biofilm: A critical review. *J Appl Oral Sci*. 2009;17(1):1-7. doi:10.1590/S1678-77572009000100002

7. Fabricius, Dahlén, G., Holm, S. E., & Möller AJ. Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand J Dent Res*. 1982;90(3):200–206. doi:10.1111/j.1600-0722.1982.tb00728.x

8. Sundqvist G. Ecology of the root canal flora. *J Endod*. 1992;18(9):427-430. doi:10.1016/S0099-2399(06)80842-3

9. Siqueira JF, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 2 - Redefining the endodontic microbiota. *J Endod*. 2005;31(7):488-498. doi:10.1097/01.don.0000157990.86638.49

10. Rôças IN, Siqueira JF. Root canal microbiota of teeth with chronic apical periodontitis. *J Clin Microbiol*. 2008;46(11):3599-3606. doi:10.1128/JCM.00431-08

11. Pinheiro ET, Gomes BPFA, Ferraz CCR, Sousa ELR, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. *Int Endod J*. 2003;36(1):1-11. doi:10.1046/j.1365-2591.2003.00603.x

12. Sakamoto, Siqueira Jr, J. F., Rôças, I. N., & Benno Y. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. *Oral Microbiol Immunol*. 2008;23(4):275–281. https://doi.org/10.1111/j.1399-302X.2007.00423.x

13. Rocas I, Siqueira J, Santos K. Association of Enterococcus faecalis with different forms of periradicular. *J Endod*. 2004;30(19):315-320.

14. Schleifer KH, Kilpper-Balz R. Transfer of Streptococcus faecalis and Streptococcus faecium to the genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. *Int J Syst Bacteriol*. 1984;34(1):31-34. doi:10.1099/00207713-34-1-31

15. Jett BD, Huycke MM, Gilmore MS. Virulence of Enterococci. *Clin Microbiol Rev*. 1994;7(4):462-478. doi:10.1128/CMR.7.4.462

16. Ørstavik, & Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Dent Traumatol*. 1990;6(4):142-149.

17. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: Its role in root canal treatment failure and current concepts in retreatment. *J Endod*. 2006;32(2):93-98. doi:10.1016/j.joen.2005.10.049

18. Love RM. Enterococcus faecalis - A mechanism for its role in endodontic failure. *Int Endod J*. 2001;34(5):399-405. doi:10.1046/j.1365-2591.2001.00437.x

19. Distel JW, Hatton JF, Gillespie MJ. Biofilm formation in medicated root canals. *J Endod*. 2002;28(10):689-693. doi:10.1097/00004770-200210000-00003

20. Evans M, Davies JK, Sundqvis G, Figdor D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. *Aust Endod J*. 2001;27(3):115. doi:10.1111/j.1747-4477.2001.tb00474.x

21. Sedgley CM, Lennan SL, Clewell DB. Prevalence, phenotype and genotype of oral enterococci. *Oral Microbiol Immunol*. 2004;19(2):95-101. doi:10.1111/j.0902-0055.2004.00122.x

22. Figdor D, Davies JK, Sundqvist G. Starvation survival, growth and recovery of Enterococcus faecalis in human serum. *Oral Microbiol Immunol*. 2003;18(4):234-239. doi:10.1034/j.1399-302X.2003.00072.x

23. Ran S, Liu B, Jiang W, Sun Z, Liang J. Transcriptome analysis of Enterococcus faecalis in response to alkaline stress. *Front Microbiol*. 2015;6(AUG):1-11. doi:10.3389/fmicb.2015.00795

24. Coolidge ED. The diagnosis and treatment of conditions resulting from diseased dental pulps. *J Nat Dent Assoc*. 1919;6:337-349. doi:10.14219/JADA.ARCHIVE.1919.0061

25. Rosenfeld EF, James GA, Burch BS. Vital pulp tissue response to sodium hypochlorite. *J Endod*. 1978;4(5):140-146. doi:10.1016/S0099-2399(78)80129-0

26. BYSTRÖM A, SUNVQVIST G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *Int Endod J*. 1985;18(1):35-40. doi:10.1111/j.1365-2591.1985.tb00416.x

27. Estrela C, Estrela CRA, Barbin EL, Spanó JCE, Marchesan MA, Pécora JD. Mechanism of action of sodium hypochlorite. *Braz Dent J*. 2002;13(2):113-117. doi:10.1590/S0103-64402002000200007

28. Greenstein G, Berman C JR. Chlorhexidine: an adjunct to periodontal therapy. *J Periodontol*. 1986;57:370-376.

29. Gomes BPFA, Souza SFC, Ferraz CCR, et al. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine rootDentinin vitro. *Int Endod J*. 2003;36(4):267-275. doi:10.1046/j.1365-2591.2003.00634.x

30. Gomes BPFA, Vianna ME, Zaia AA, Almeida JFA, Souza-Filho FJ, Ferraz CCR. Chlorhexidine in endodontics. *Braz Dent J*. 2013;24(2):89-102. doi:10.1590/0103-6440201302188

31. White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine. *J Endod*. 1997;23(4):229-231. doi:10.1016/S0099-2399(97)80052-0

32. Leonardo MR, Tanomaru Filho M, Silva LAB, Nelson Filho P, Bonifácio KC, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. *J Endod*. 1999;25(3):167-171. doi:10.1016/S0099-2399(99)80135-6

33. Dametto FR, Ferraz CCR, De Almeida Gomes BPF, Zaia AA, Teixeira FB, De Souza-Filho FJ. In vitro assessment of the immediate and prolonged antimicrobial action of chlorhexidine gel as an endodontic irrigant against Enterococcus faecalis. *Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology*. 2005;99(6):768-772. doi:10.1016/j.tripleo.2004.08.026

34. Khademi A ali, Mohammadi Dr Z, Havaee A. Evaluation of the antibacterial substantivity of several intra-canal agents. *Aust Endod J*. 2006;32(3):112-115. doi:10.1111/j.1747-4477.2006.00033.x

35. Komorowski R, Grad H, Wu XY, Friedman S. Antimicrobial substantivity of chlorhexidine-treated bovine root dentin. *J Endod*. 2000;26(6):315-317. doi:10.1097/00004770-200006000-00001

36. Rosenthal S, Spångberg L, Safavi K. Chlorhexidine substantivity in root canal dentin. *Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology*. 2004;98(4):488-492. doi:10.1016/j.tripleo.2003.07.005

37. Önçağ Ö, Hoşgör M, Hilmioğlu S, Zekioğlu O, Eronat C, Burhanoğlu D. Comparison of antibacterial and toxic effects of various root canal irrigants. *Int Endod J*. 2003;36(6):423-432. doi:10.1046/j.1365-2591.2003.00673.x

38. Krithikadatta J, Indira R, Dorothykalyani AL. Disinfection of Dentinal Tubules with 2% Chlorhexidine, 2% Metronidazole, Bioactive Glass when Compared with Calcium Hydroxide as Intracanal Medicaments. *J Endod*. 2007;33(12):1473-1476. doi:10.1016/j.joen.2007.08.016

39. Okino LA, Siqueira EL, Santos M, Bombana AC, Figueiredo JAP. Dissolution of pulp tissue by aqueous solution of chlorhexidine digluconate and chlorhexidine digluconate gel. *Int Endod J*. 2004;37(1):38-41. doi:10.1111/j.1365-2591.2004.00749.x

40. Jeansonne MJ. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. *J Endod*. 1994;20(6):276-278. doi:10.1016/S0099-2399(06)80815-0

41. Debelian G, Trope M. Cleaning the third dimension. *Endod Prac*. 2015;8(6):18-21. http://www.schottlander.com/docs/default-source/dfu-pdf's/article---cleaning-the-third-dimension.pdf?sfvrsn=2

42. Bao P, Shen Y, Lin J, Haapasalo M. In Vitro Efficacy of XP-endo Finisher with 2 Different Protocols on Biofilm Removal from Apical Root Canals. *J Endod*. 2017;43(2):321-325. doi:10.1016/j.joen.2016.09.021

43. Azim AA, Aksel H, Zhuang T, Mashtare T, Babu JP, Huang GTJ. Efficacy of 4 Irrigation Protocols in Killing Bacteria Colonized in Dentinal Tubules Examined by a Novel Confocal Laser Scanning Microscope Analysis. *J Endod*. 2016;42(6):928-934. doi:10.1016/j.joen.2016.03.009

44. Elnaghy AM, Mandorah A, Elsaka SE. Effectiveness of XP-endo Finisher, EndoActivator, and File agitation on debris and smear layer removal in curved root canals: a comparative study. *Odontology*. 2017;105(2):178-183. doi:10.1007/s10266-016-0251-8

45. Alves FRF, Andrade-Junior C V., Marceliano-Alves MF, et al. Adjunctive Steps for Disinfection of the Mandibular Molar Root Canal System: A Correlative Bacteriologic, Micro–Computed Tomography, and Cryopulverization Approach. *J Endod*. 2016;42(11):1667-1672. doi:10.1016/j.joen.2016.08.003

46. Jou YT, Karabucak B, Levin J, Liu D. Endodontic working width: Current concepts and techniques. *Dent Clin North Am*. 2004;48(1):323-335. doi:10.1016/j.cden.2003.12.006

47. Haapasalo M, Ørstavik D. In vitro Infection and Disinfection of Dentinal Tubules. *J Dent Res*. 1987;66(8):1375-1379. doi:10.1177/00220345870660081801

48. Marinho ACS, Martinho FC, Gonçalves LM, Rabang HRC, Gomes BPFA. Does the Reciproc file remove root canal bacteria and endotoxins as effectively as multifile rotary systems? *Int Endod J*. 2015;48(6):542-548. doi:10.1111/iej.12346

49. Goud S, Aravelli S, Dronamraju S, Cherukuri G, Morishetty P. Comparative Evaluation of the Antibacterial Efficacy of Aloe Vera, 3% Sodium Hypochlorite, and 2% Chlorhexidine Gluconate Against Enterococcus faecalis: An In Vitro Study. *Cureus*. 2018;10(10). doi:10.7759/cureus.3480

50. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. *J Endod*. 2014;40(4):509-514. doi:10.1016/j.joen.2013.11.026

51. OA. P. Current challenges and concepts in the preparation of root canal systems: a review. *J Endod*. 2004;Aug;30(8):559-67.

https://doi.org/10.1097/01.DON.0000129039.59003.9D

52. Versiani MA, Leoni GB, Steier L, et al. Micro-computed tomography study of oval-shaped canals prepared with the self-adjusting file, reciproc, waveone, and protaper universal systems. *J Endod*. 2013;39(8):1060-1066. doi:10.1016/j.joen.2013.04.009

53. De-Deus G, Barino B, Zamolyi RQ, et al. Suboptimal debridement quality produced by the single-file F2 protaper technique in oval-shaped canals. *J Endod*. 2010;36(11):1897-1900. doi:10.1016/j.joen.2010.08.009

54. Wu MK, Dummer PMH, Wesselink PR. Consequences of and strategies to deal with residual post-treatment root canal infection. *Int Endod J*. 2006;39(5):343-356. doi:10.1111/j.1365-2591.2006.01092.x

55. Kulasekara BR, Kamischke C, Kulasekara HD, Christen M, Wiggins PA, Miller SI. c-di-GMP heterogeneity is generated by the chemotaxis machinery to regulate flagellar motility. *Elife*. 2013;2013(2):1-19. doi:10.7554/eLife.01402

56. Carvalho MC, Zuolo ML, Arruda-Vasconcelos R, et al. Effectiveness of XP-Endo Finisher in the reduction of bacterial load in oval-shaped root canals. *Braz Oral Res*. 2019;33:1-8. doi:10.1590/1807-3107BOR-2019.VOL33.0021

57. Zamany A, Safavi K, Spångberg LSW. The effect of chlorhexidine as an endodontic disinfectant. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2003;96(5):578-581. doi:10.1016/S1079-2104(03)00168-9

58. Siqueira JF, Rôças IN, Santos SRLD, Lima KC, Magalhães FAC, De Uzeda M. Efficacy of instrumentation techniques and irrigation regimens in reducing the bacterial population within root canals. *J Endod*. 2002;28(3):181-184. doi:10.1097/00004770-200203000-00009

59. Athanassiadis B, Abbott P V., Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. *Aust Dent J*. 2007;52(1 SUPPL.). doi:10.1111/j.1834-7819.2007.tb00527.x

60. Emilson. Susceptibility of various microorganisms to chlorhexidine. *Andin J Dent Res*. 1977;85(4):255-265. htps://doi.org/10.1111/j.1600-0722.1977.tb00561.x

61. Sheldon AT. Antiseptic “resistance”: Real or perceived threat? *Clin Infect Dis*. 2005;40(11):1650-1656. doi:10.1086/430063

62. Lee JK, Baik JE, Yun CH, et al. Chlorhexidine Gluconate Attenuates the Ability of Lipoteichoic Acid from Enterococcus faecalis to Stimulate Toll-like Receptor 2. *J Endod*. 2009;35(2):212-215. doi:10.1016/j.joen.2008.10.018

63. Zehnder M. Root Canal Irrigants. *J Endod*. 2006;32(5):389-398. doi:10.1016/j.joen.2005.09.014

64. Basrani BR, Manek S, Sodhi RNS, Fillery E, Manzur A. Interaction between Sodium Hypochlorite and Chlorhexidine Gluconate. *J Endod*. 2007;33(8):966-969.

doi:10.1016/j.joen.2007.04.001

65. Huff JE, Haseman JK, Elwell MR. CARCINOGENICITY OF p-CHLOROANILINE IN RATS A N D MICE. 1991;29(2):119-124.

66. Vivacqua-Gomes N, Ferraz CCR, Gomes BPFA, Zaia AA, Teixeira FB, Souza-Filho FJ. Influence of irrigants on the coronal microleakage of laterally condensed gutta-percha root fillings. *Int Endod J*. 2002;35(9):791-795. doi:10.1046/j.1365-2591.2002.00569.x