



**SATHYABAMA INSTITUTE OF
SCIENCE & TECHNOLOGY**

(Deemed to be University)

Jeppiar Nagar, Chennai



**EVALUATING THE EFFECTIVENESS OF THREE
DIFFERENT IRRIGANT AGITATION DEVICES IN
ERADICATING *E. FAECALIS* BIOFILM IN HUMAN
MANDIBULAR PREMOLARS: AN *IN VITRO* STUDY.**

By

Dr. DHANIREDDY V PADMAJA

Dissertation submitted to
Sathyabama Institute of Science & Technology
(Deemed to be University) Chennai, Tamil Nadu.

In partial fulfillment of the requirements for the degree of

Master of Dental Surgery

In the Speciality of

CONSERVATIVE DENTISTRY AND ENDODONTICS

Under the guidance of

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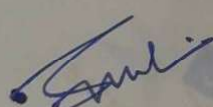
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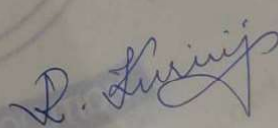
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A Dissertation submitted to the **Sathyabama Institute of Science & Technology (Deemed to be University)** Chennai, Tamil Nadu, in partial fulfillment of the requirements for the degree of **Master of Dental Surgery in the Speciality of Conservative Dentistry and Endodontics**.

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To,
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I am pleased to inform you that the Institutional Human Ethics Committee (IHEC) has approved your project "**Evaluating the Effectiveness of three different irrigant Agitation devices in eradicating *E. faecalis* biofilm in Human mandibular premolars: An In Vitro Study**" based on the presentation of the proposal made and recommendations of the IHEC committee meeting held on 30/10/2018.

The validity of this ethical clearance is for only one year till 30/10/2019 and in case if the study is not completed within the stipulated time they have to apply to renew for ethical clearance. You are expected to submit six monthly progress and final report of the study to the IHEC.

Thanking you,

Dr. Thayumanvan

Deputy Chairperson

DECLARATION OF PLAGIARISM CHECK

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Yours sincerely,

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Introduction	1%
Review of Literature	3%
Materials and Methods	1%
Results and Discussion	5%
Summary	1%
Conclusion	100 % original

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INTRODUCTION

Pulpal and periapical diseases occur due to microorganisms invading into dental hard tissues and their further progression into the root canal system. ⁽¹⁾ The primary goal of chemo-mechanical preparation during root canal treatment is to eliminate or reduce bacterial load in the root canal system that can allow healing of periradicular tissues with a positive treatment outcome. ⁽²⁾ Microbes are present in almost all parts of the root canal system, including fins, isthmus, and anastomoses. It may be found at varying depths of up to 300 μm within the dentinal tubules from the root canal lumen. ⁽³⁾ There is regional variation in the depth to which bacteria invade dentine; cervical tubules are invaded to a greater depth than the mid-root tubules, which are, in turn, invaded more than those in the apical region. ⁽⁴⁾ The mean number of tubules at any given age in coronal, cervical, and mid-root dentin are similar; however, there are significantly fewer dentinal tubules/area in apical dentin resulting in varying bacterial penetration between areas of the root. The relationship of the size of the bacteria to the tubule will influence its ability to penetrate. A large size of the bacterium relative to the tubule's size will limit the bacteria's ability to penetrate the tapering tubule from the pulpal side. ⁽⁵⁾

Studies have shown the prevalence of certain species in teeth with post-treatment infection, such as *Enterococci*, *Streptococci*,

Lactobacilli, *Actinomyces* and fungi such as *Candida*.⁽⁶⁾ *Enterococcus faecalis* (*E. faecalis*) is of interest among different bacterial species because it is the most commonly isolated species from root canals presenting with post-treatment infection, with prevalence ranging from 24% to 77%.⁽⁷⁾ *Enterococcus faecalis* is a gram-positive, facultative anaerobe that can occur singly, in pairs, or as short chains. *E. faecalis* possess various survival and virulence factors, including its ability to compete with other microorganisms, invade dentinal tubules, and resist nutritional deprivation where it can survive for a prolonged period under adverse conditions such as starvation, high pH of sodium hypochlorite and calcium hydroxide medication.⁽⁸⁾ *E. faecalis* is the only species that has been widely studied for its capacity to form biofilms. *E. faecalis* also has the ability to form a biofilm that helps it resist destruction and become more resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm-producing organisms.⁽⁹⁾

During non-surgical root canal treatment, it is not always easy to thoroughly shape and clean the root canal system due to its intricate nature. During cleaning and shaping, most of the nickel-titanium instruments are unable to plane more than 60%⁽¹⁰⁾ of the root canal surface or instrument only the canal's central body, leaving canal fins, isthmi, and cul-de-sacs untouched after completion of the preparation.⁽¹¹⁾ These areas might harbor tissue debris, microbes, and their by-products, which might prevent close

adaptation of the obturation material, compromising the seal and eventually resulting in persistent periradicular inflammation. ⁽¹²⁾ Therefore, irrigation procedure plays a vital part in root canal debridement because it allows cleaning of root canal system beyond what could be achieved by instrumentation alone. ⁽¹³⁾

Irrigants exert their effects by physical, chemical and biological actions. On the physical front, streaming forces are delivered to the canal walls. On the chemical front, the active components exert specific actions on the organic and inorganic debris. On the biological front, they exert the antimicrobial action on the organisms within the canal by inactivating or killing them. Root canals are considered as "closed systems" where the fluid dynamics of the irrigant play a significant role in ensuring optimal actions. ⁽¹⁴⁾ In contemporary endodontic practice, dual irrigation solutions such as Sodium hypochlorite (NaOCl) with Ethylene diamine tetra-acetic acid (EDTA) are commonly used to complement the shortcomings that are associated with the use of a single irrigant. ⁽¹⁵⁾ More importantly, these irrigants must be brought into direct contact with the entire canal surface for their effective action. This is particularly more challenging in the apical portion of root canals. ⁽¹⁶⁾ Therefore in order to achieve this, agitation of an irrigant becomes necessary and could help in better irrigant dispersal into the entirety of the root canal system. ⁽¹⁷⁾

Several agitation techniques^{(18), (19), (20)} were proposed to improve the penetration of the irrigant into the complexities of root canal system to enhance the contact of irrigant's solution with the surface of canal walls, particularly the apical portions of root canals, isthmuses and dentinal tubules.

To ensure maximum disinfection and to achieve desired results, different agitation techniques were followed and compared in this study. EndoActivator (Dentsply Sirona) is a cordless, battery-operated sonic handpiece that uses noncutting polymer tips to quickly and vigorously agitate irrigant solutions during treatment⁽²¹⁾. The EndoActivator tips have a simple, easy snap-on/snap-off design and are color-coded as yellow, red, and blue to approximately correspond to file sizes #20/.02, #25/.04, #30/.06, respectively. These tips are made from a noncutting, medical-grade polymer that are strong and flexible and are 22 mm long with orientational depth gauge rings positioned at 18, 19, and 20 mm. EndoActivator polymer tips are strong and flexible, and will not cut dentin, and as such, will not ledge, apically transport, or perforate a canal..⁽²²⁾

Ultrasound is used in Endodontics to complement the cleaning ability of root canal irrigants. Passive ultrasonic irrigation (PUI) was first described by Weller et al. ⁽²³⁾ PUI relies on transmitting acoustic energy from an oscillating file or smooth wire to an

irrigant solution in the canal. The energy is transmitted through ultrasonic waves that can induce acoustic streaming and cavitation of the irrigant. ⁽²⁴⁾ After shaping the root canal to master apical file size (irrespective of the preparation technique used), a small file or smooth wire is introduced in the root canal, as close as possible to the apical region. The root canal is then flooded with an irrigant, and the ultrasonic file agitates the irrigant solution. As the root canal has already been instrumented till the master apical file, the ultrasonic file or smooth wire can move freely, and the irrigant can penetrate more easily into the apical part of the root canal system. ⁽²⁵⁾

XP-Endofinisher (XPF) (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) is an adjunctive approach that improves the effectiveness of irrigation in endodontics. XPF is an ISO 25/.00 instrument produced using a particular type of alloy, the NiTi Max Wire (Martensite-Austenite Electropolish-FleX, FKG). The file is straight in its M phase when it is cooled, and it will change to an A phase on exposure to body temperature, where it will have its unique spoon shape design with a length of 10 mm from the tip and have a depth of 1.5 mm because of its molecular memory according to the manufacturer. ⁽²⁶⁾

Research has shown that different procedures such as histologic sections, scanning electron microscope observation, and microbiological analyses [colony forming units (CFU) + molecular

methods] at different root canal levels can be routinely done to identify *E. faecalis*.^{(27),(28)} It is well known that molecular methods are more sensitive than culture methods, but they do have their own limitations such as overestimating the microbial load due to its inability to differentiate live from dead bacterial remnants, with the real time polymerase chain reaction (PCR) being an exception. They are also laborious and expensive. Thus colony-forming units of cultivable bacteria, was used as a quantitative analysis to evaluate dentin infection. However, this method does not give clear information about the spatial distribution of bacteria inside the dentin.^{(29),(30)}

Confocal LASER scanning microscope (CLSM) helps us to visualize the endodontic microflora by producing high-resolution images and thereby allows us to study the spatial distribution of microorganisms. It is commonly used with vital staining techniques to determine the viability profile and architecture of the biofilm.⁽³¹⁾

Numerous studies have evaluated reduced bacterial load after using various irrigant agitation systems^{(32), (33)}. But most of the studies have evaluated biofilm removal efficacy by using only one of the methods like colony forming units,^{(34), (35), (36)} molecular methods (PCR), scanning electron microscope or histological sections.^{(37), (38), (39)} Numerous studies have prepared the root canal to master apical file size before inoculation of *E.faecalis* and

performed only irrigant agitation techniques post incubation, which does not simulate the clinical condition. (2), (40), (41) There are limited studies evaluating the biofilm removal efficacy in the main canal as well as in dentinal tubules and there is a lack of data comparing the effectiveness of three different irrigation agitation devices, namely Passive ultrasonic irrigation, Endoactivator, and XP-Endofinisher, in eradicating biofilm quantitatively and qualitatively.

AIM AND OBJECTIVES

AIM:

The aim of this study is to quantitatively and qualitatively evaluate the effectiveness of three different irrigant agitation devices in eradicating endodontic biofilm from root canals and from the dentinal tubules of extracted single rooted human mandibular premolars.

OBJECTIVE:

1. To evaluate the effectiveness of different irrigant agitation devices namely EndoActivator, passive ultrasonic irrigation and XP-Endofinisher in eradicating *E. faecalis* biofilm from the root canals by quantifying the cultivable bacteria using colony-forming units,
2. To compare the bacterial viability in root canals treated by three different irrigant agitation devices by directly visualizing the live/dead bacteria within the dentinal tubules using confocal laser scanning microscopy.

HYPOTHESIS:

1. The null hypothesis is that there would be no statistically significant difference in eradicating *E. faecalis* biofilm from the root canals and dentinal tubules between XP-Endofinisher, passive ultrasonic irrigation and EndoActivator.
2. The alternate hypothesis is that there is a statistically significant difference between XP-Endofinisher, passive ultrasonic irrigation and EndoActivator in eradicating *E. faecalis* biofilm from the root canals and in dentinal tubules.

REVIEW OF LITERATURE

E. Faecalis biofilm:

E T Pinheiro et al., 2003 ⁽⁴²⁾ evaluated the microbiota of 30 root-filled teeth with persisting periapical lesions and tested the antibiotic susceptibility of the most prevalent species. Fifty-five bacterial species were isolated, 80% were gram-positive, and 58% facultative anaerobic microorganisms. The bacterial genera most frequently recovered were *Enterococcus*, *Streptococcus*, *Peptostreptococcus* and *Actinomyces*. Antibiotic sensitivity of *Enterococcus faecalis* and *Peptostreptococcus* spp. was accomplished with the E-test system. All species studied were susceptible to benzyl-penicillin, amoxicillin, amoxicillin combined with clavulanate. However, 20% of the *E. faecalis* strains were resistant to erythromycin and 60% to azithromycin. They concluded that *E. faecalis* was the most frequently recovered bacteria found in 11(45.8%) of the 24 canals with bacteria.

Jose F. Siqueira et al., 2004 ⁽⁴³⁾ investigated the occurrence of several microbial species in cases of failed endodontic treatment utilizing the polymerase chain reaction (PCR). Twenty-two root-filled teeth with persistent periradicular lesions were selected for retreatment, and samples from the root canals were taken from those teeth. DNA was extracted from the root canal samples and analyzed for 19 microbial taxa by using PCR. This study showed that *Enterococcus faecalis* was the most prevalent species seen in 77%

of the cases. The other most prevalent species were *Propionibacterium propionicum* (52%), *Pseudoramibacter alactolyticus* (52%), *Dialister pneumosintes* (48%), and *Filifactor alocis* (48%). *Candida albicans* were found in 9% of the samples. They concluded that microorganisms were seen in all cases of root canal-filled teeth with periradicular lesions, which strongly supports the assertion that treatment failures are more of an infectious etiology, caused by persistent or secondary intraradicular infections. *E. faecalis* was the foremost prevalent species, followed by four other anaerobic species: *P. alactolyticus*, *P. propionicum*, *D. pneumosintes*, and *F. alocis*.

Kayaoglu et al., 2004 ⁽⁴⁴⁾ in their review article, discussed the *E. faecalis* virulence factors related to endodontic infection and the periradicular inflammatory response. The most important virulence factors are bacterial surface adhesins, sex pheromones, aggregation substance, lipoteichoic acid, extracellular superoxide production, and the release of two crucial lytic enzymes: gelatinase and hyaluronidase. They also promote a binding relationship between *E. faecalis* and various eukaryotic cells, strengthen the adherence of bacteria to type I and IV collagen, and act as a protective factor against the host's neutrophils. On the other hand, the surface adhesives add to the virulence of *E. faecalis* by attaching bacteria to several essential substances, such as abiotic surfaces (for biofilm), other bacterial species (for gene and nutrient exchange), collagen fibers, human serum and dental tissues.

Isabela N Roca et al., 2004 ⁽⁴⁵⁾ stated that *E. faecalis* was related to different periapical disease forms, including primary endodontic infections and persistent infections. *E. faecalis* is more frequently associated with asymptomatic chronic periradicular lesions than with acute periradicular periodontitis or acute periradicular abscesses in case of primary endodontic infections. It was seen in 4 - 40% of primary endodontic infections but was found to be much higher in persistent periradicular lesions. Failed root canal therapy more likely contained *E. faecalis* nine times more than primary endodontic infections.

Christine Sedgley et al., 2006 ⁽⁴⁶⁾ evaluated the prevalence of *E. faecalis* in root canals. In this study, culture and real-time quantitative polymerase chain reaction (qPCR) were compared to detect and quantify *E. faecalis* in the same root canal sample. Root canal samples obtained from primary infection (n = 40) and retreatment (n = 48) cases were divided into two equal aliquots that were independently analyzed using culture and qPCR. This study showed that *E. faecalis* was detected in 10.2% and 79.5% of samples by culture and qPCR, respectively. *E. faecalis* was detected more in retreatment than primary infection samples (89.6% versus 67.5%).

Narayanan LL and Vaishnavi C et al., 2010 ⁽⁴⁷⁾ discussed the resistance mechanism of *E. faecalis* in their review article. *E. faecalis* are gram-positive cocci and facultative anaerobes. They are typical intestinal organisms and may occupy the gingival sulcus and oral cavity. When it is present in small numbers, it can be easily

eliminated; but it is difficult to eradicate it in large numbers. *E. Faecalis* has many distinct attributes that make it an outstanding root canal survivor. The following may be carried out by such microorganisms.

It can persist in poor nutrient environmental conditions.

It can thrive in the presence of several medications (e.g., calcium hydroxide) and irrigants (e.g., sodium hypochlorite).

Forms biofilm in medicated root canals.

Invade and metabolize fluids in the dentinal tubules and adhere to collagen.

Convert into a viable but non-cultivable state.

It can acquire antibiotic resistance.

Survive in extreme conditions with low pH, high salinity and high temperatures.

Endure prolonged periods of starvation and utilize tissue fluid that flows from the periodontal ligament.

Kapil Jhajharia et al., 2015 ⁽⁴⁸⁾ in their review article, discussed about resistance mechanisms of *E. faecalis*. *Enterococci* are facultative anaerobes that can grow in the presence or absence of oxygen. They can grow in highly alkaline pH, salt-concentrated environments, temperatures between 10 and 45°C, and survive at a temperature of 60 degrees Celsius for 30 minutes. *E. faecalis* may suppress the action of lymphocytes, potentially contributing to

endodontic failure. *E. faecalis* can withstand calcium hydroxide intracanal dressing for more than ten days by forming a biofilm that helps to resist destruction by allowing the bacteria to become 1000 times more resistant to antibodies, phagocytosis, and antimicrobials than non-biofilm-producing organisms. Ca(OH)_2 , a commonly used intracanal medicament, may be ineffective in killing *E. faecalis* on its own if a high pH level is not maintained. *E. faecalis* may form a biofilm that can withstand the dressing of calcium hydroxide by maintaining pH homeostasis. *E. faecalis* was found difficult to survive at a pH of 11.5 or more.

Gijo john et al., 2015 ⁽⁴⁹⁾ in their review article, explained about adhesion of *E. faecalis* to the dentinal tubule. *E. faecalis* can penetrate dentinal tubules, sometimes to a profound extent. Collagen is widely considered the primary substrate for specific binding of *E. faecalis* to dentine. The collagen-binding protein of *E. faecalis* (Ace) and a serine protease (Spr) have been proposed to play significant roles in binding to the root canal wall. Ace also promotes *E. faecalis* binding to collagen type I, and in vitro ace gene expression at 37⁰C was enhanced in the presence of collagen. The ability of *E. faecalis* to grow as chains has been suggested as another explanation for the moderate to the great extent of tubule invasion. After initial attachment to the poorly non-mineralized pre-dentine at the tubule orifices, deeper penetration may not require specific binding as invasion may result from intratubular cell growth. This property can enable this species to escape from the

action of endodontic instruments and irrigants used during chemomechanical preparation.

Studies in Biofilm Models:

Kishen et al., 2012 ⁽⁵⁰⁾ review article explained various factors to be considered in Biofilm Model Systems. The antimicrobial resistance observed in biofilm bacteria is not primarily due to the classic genetic mechanisms; instead, this arises due to certain peculiarities associated with the biofilm growth. The types of resident bacterial species, nature of bacterial adherence to the substrate, physicochemical characteristics of the substrate, thickness of the biofilm, bacterial cell density, amount of extracellular polymeric substances (EPS), and phenotypical/genotypical modification of the resident bacteria are all features that could contribute to antimicrobial resistance in biofilm bacteria. Typically, different antimicrobial resistance mechanisms may act concurrently or synergistically in a biofilm. Understanding some of these mechanisms is critical in developing biofilm model systems for different applications in endodontics.

Rajshekhar Chatterjee et al., 2015 ⁽⁵¹⁾ compared manual dynamic agitation and sonic agitation to remove intra radicular *E. faecalis*. Extracted mandibular premolars were sectioned at the cervical level and divided into three groups ($n = 30$). Root canals were instrumented up to F4 Protaper. Roots were sterilized, and *E. faecalis* bacteria were inoculated and incubated in the root canal space for four weeks. Biofilm was confirmed using scanning

electron microscopy and Gram staining. All groups were irrigated with a side vented needle using 3% sodium hypochlorite (NaOCl) for 60 seconds. Two experimental groups were agitated with manual dynamic agitation (with master gutta-percha cone) and sonic agitation (EndoActivator). The remaining bacteria were collected using sterile paper points, which were incubated inside brain-heart infusion (BHI) broth to check for colony-forming units. Experimental groups (endoactivator and manual dynamic agitation) showed a significant difference in their mean colony count compared with the control group, with $P < 0.001$. They concluded sonic agitation with EndoActivator has proven to be the best irrigating system followed by manual dynamic agitation and conventional needle irrigation.

Wajih Hage et al., 2019 ⁽³⁴⁾ assessed the antibacterial action of sonic, ultrasonic, and laser-activated irrigation and 5.25% sodium hypochlorite (NaOCl) against *Enterococcus faecalis* in an infected tooth. Forty-four extracted mandibular premolars were mechanically prepared, sterilized, and inoculated with *E. faecalis* for one week. Bacterial counts after inoculation were evaluated in 4 randomly chosen teeth, and the remaining root canals were divided into four groups. Group A: laser-activated Irrigation by photon-induced photoacoustic streaming, Group B: sonic Irrigation by EDDY, Group C: ultrasonic Irrigation by EndoUltra, and Group D: 5.25% NaOCl. Colony-forming unit (CFU) counts were measured. The study results showed no statistically significant differences

regarding the decrease in CFUs in the root canals activated by EDDY, EndoUltra and PIPS.

Mohammad I. Al-Obaida et al., 2019 ⁽⁵²⁾ evaluated various irrigant agitation devices' ability in eradicating *Enterococcus faecalis* from the dentinal tubules of human teeth. Extracted human teeth (n=50) were instrumented with a 0.04 taper to size 30 K. The roots were autoclaved, and *E. faecalis* was inoculated. The root canals were allocated to one of the four intervention groups and disinfected with (A) standard needle irrigation, (B) EndoUltra Ultrasonic Activator, (C) EndoActivator system, (D) EDDY sonic activation, and (E) saline-treated group, and (F) group without bacterial inoculation. The roots were cut in half, dyed with a LIVE/DEAD Back Light Bacterial Viability Package, and then scanned under confocal laser scanning microscopy (CLSM) to identify live and dead bacteria in the dentinal tubules. CLSM images showed variations among the groups. Compared to standard needle irrigation, which had more live bacteria, both the EndoUltra Ultrasonic Activator group and the EDDY group had a mix of dead and live bacteria. In contrast, the EndoActivator group had mostly dead bacteria. Overall, in reducing live bacteria inside the root canal, the EndoActivator method was found to be superior to all other techniques.

Karina Gonzalez Camara Fernandes et al., 2020 ⁽⁵³⁾ analyzed the effectiveness of three different irrigation systems: EndoActivator, passive ultrasonic irrigation (PUI), and Easy Clean to reduce *Enterococcus faecalis*, after instrumentation with the reciprocating

system, through microbiological collection and culture method. Sixty extracted human lower premolars were taken with a length standardized to 16 mm. The access cavity was prepared, inoculated with *E. faecalis*, and incubated for 21 days at 37°C. Initial samples (S1) were taken with an absorbent paper point to confirm the contamination, and then teeth were instrumented with Wave One Primary. Teeth were divided into four groups consistent with the final irrigation protocol ($n = 15$): group 1 – EndoActivator, group 2 – PUI, group 3 - Easy Clean, and group 4 - control group irrigated with sterile saline solution and without any agitation. Following instrumentation of the canals, the agitation protocol was three cycles of 20 seconds of sodium hypochlorite, three cycles of 17% EDTA, and three cycles of hypochlorite according to each system. After the chemomechanical preparation and agitation of the irrigating solutions, the final collections (S2) for counting the colony-forming units (CFU/mL) were taken. The Control group presented a significantly higher CFU/mL after the chemical-mechanical preparation than the other groups, which were similar to each other.

Inoculation:

R. C. D. Swimberghe et al., 2018 ⁽⁵⁴⁾ in their review article, discussed about inoculation of bacterial samples in the root canal. It states the inoculum determines the biofilm's starting population. The inoculum cell number should be high enough to allow enough cells to adhere to the surface and begin the biofilm formation. When

the inoculum suspension is introduced into the root canal, bacterial growth occurs only inside the root canal. To prevent air bubbles inclusion in the root canal, care should be taken so that inoculum is introduced as close as possible to the apical canal terminus, thus filling the root canal from apical to coronal. In this manner, the bacterial suspension contacts the entire root canal wall, along which a biofilm can form. When quantifying the intracanal biofilm after endodontic treatment, care needs to be taken not to include the extra canal biofilm in the quantification, which could occur if the entire tooth gets exposed to the inoculum. This review paper also discussed the inoculum concentration and found that the concentration varied between 10^4 and 10^{10} CFU mL⁻¹. A concentration of 10^8 CFU mL⁻¹ was most frequently used (38% of the studies).

Substrate material:

Carlos H. R. Camargo et al., 2007 ⁽⁵⁵⁾ evaluated the number and the diameter of dentin tubules in root canals of human and bovine teeth at the cervical, middle, and apical thirds. Twenty-four single-rooted extracted human premolars were divided into four groups (n = 6): GH1 - 10 to 15 years, GH2 - 16 to 30 years, GH3 - 31 to 45 years, and GH4 - 46 to 80 years; and 24 bovine incisors were divided into four groups (n = 6): GB1, central; GB2, first lateral; GB3, second lateral; and GB4, third lateral. The crowns were removed from the specimens, then debrided, sectioned longitudinally in the buccolingual direction, and then subjected to

ultrasonic cleaning. Sectioned specimens were examined under a scanning electron microscope at 1,000x and 5,000x magnification. They concluded that the cervical third of the root presented the highest mean values related to the dentin tubule's number and diameter, followed by the middle and apical thirds, for both specimens studied. In contrast to human specimens, bovine specimens had a slightly higher mean value concerning the number of dentin tubules; however, there were no statistically significant variations between the two samples concerning dentin tubules' diameter.

Kakoli et al., 2009 ⁽⁵⁶⁾ evaluated the effect of age on the prevalence and depth of bacterial penetration inside dentinal tubules. Fifty-six single-rooted teeth were divided into two groups based on the patient's age: young (ages 18 –25) and old (age greater than or equal to 60). The teeth were prepared and inoculated with a standardized load of bacteria and incubated for 20 days. Histological evaluation was performed to determine the degree of dentinal tubules infection by counting the number of invaded tubules and the penetration depth of bacteria inside the tubules. A higher number of infected dentinal tubules and deeper bacterial penetration were found in the young teeth than the old teeth. This may be due to more sclerotic dentinal tubules in the old dentin at any root level. A difference in the number of infected dentinal tubules may be clinically relevant because it may impact dentin's ability to harbor bacteria after

endodontic infection and the ability of clinical procedures to disinfect the canal wall.

Ozdemir HO et al., 2010 ⁽⁵⁷⁾ evaluated the effects of ethylene diamine tetracetic acid (EDTA) and sodium hypochlorite (NaOCl) on *E. faecalis* biofilm growth in root canal dentin of young and old individuals. Root canals of extracted single-rooted human teeth, both young (<30 years) and old (>60 years) teeth, have been sectioned at the level of the crown and the apical sections. With #2 Gates-Glidden burs, the root canals of the mid-root portions were enlarged. The samples were then incubated in *E. faecalis* suspension for 24-hour following treatment with 17 percent EDTA + 2.5 percent NaOCl, 17 percent EDTA alone, 2.5 percent NaOCl alone, or saline. After that, with # 3 Gates- Glidden burs, root canal samples were enlarged again, and the removed dentin chips were collected. Bacteria were dispersed using sonication, diluted serially, and then plated for counting on agar plates for colony-forming units. To analyze biofilm formation on the dentin, Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) experiments were also conducted. This study showed that the combination of EDTA and NaOCl significantly reduced the amount of intracanal biofilm in both age groups. However, the *E. faecalis* counts in the old group were still higher ($P < .05$). It is unclear whether the differences in bacterial adhesion are due to age-induced changes in the dentin or the formation of more smear layers. The authors assumed that the increased

adherence of *E. faecalis* in older teeth might be due to the dentin material.

Substrate sterilization:

William g. Kohn et al., 2003 ⁽⁵⁸⁾ Centers for Disease Control and Prevention, or CDC proposed Guidelines for infection control in dental health care settings 2003. Extracted teeth are usually collected for use in preclinical academic training. These teeth should be cleaned to remove blood and debris and maintained in a hydrated state in a well-constructed closed container during transport and labelled with a biohazardous symbol. Using a cost-effective storage solution (e.g., Saline or water) is practical as these teeth will be autoclaved before clinical exercises or study. Liquid germicides can also be used, but they do not reliably disinfect interior pulp tissue and external surface. Teeth should be heat-sterilized to allow for safe handling before being used in an educational setting. Microbial growth can be eliminated using an autoclave cycle for forty minutes, but students should still follow standard precautions because preclinical exercises simulate clinical cases. Autoclaving teeth for preclinical exercises do not alter their physical properties sufficiently to compromise the learning experience. However, it is unknown whether autoclave sterilization of extracted teeth affects dentinal structure to the point that the chemical and micro-chemical relationship between dentin and dental materials would be affected for research purposes on dental

materials. Using teeth that do not contain amalgam is preferred in educational settings because they can be safely autoclaved. Extracted teeth containing amalgam restorations shouldn't be heat-sterilized due to the potential hazard from mercury vaporization and exposure. Suppose extracted teeth containing amalgam restorations are to be used; immersion in a 10% formalin solution for two weeks should be effective in disinfecting both the internal and external surfaces of the teeth. If using formalin, manufacturer Material Safety Data Sheet (MSDS) should be reviewed for occupational safety and health concerns and to ensure compliance with Occupational Safety and Health Administration (OSHA) regulation.

Incubation time:

Sonja Stojicic et al., 2013 ⁽⁵⁹⁾ assessed the effect of the source of biofilm bacteria, the extent of biofilm maturation, and also the type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. Multispecies biofilms from six donors' plaque bacteria were grown on collagen-coated hydroxyapatite disks for up to eight weeks. After 1, 2, 3, 4, and 8 weeks of growth, the biofilms were exposed to 1% sodium hypochlorite (Naocl), 0.2/0.4% iodine-potassium iodide (IPI), and 2% chlorhexidine (CHX) for 1 and 3 minutes. The percentage of bacterial biofilm killed was determined using LIVE/DEAD viability staining and confocal laser scanning microscopy. The current study results showed that 1 and 2 week-old biofilms were very or moderately sensitive to the disinfecting agents used in this study which killed 20%–100% of the biofilm

bacteria. After three weeks of growth, the biofilm bacteria were more immune against the same disinfecting agents and killed only 10%–30% of the bacteria. 1% NaOCl, 0.2/0.4% IPI, and 2% CHX effectively killed 1- and 2-week-old biofilm bacteria, whereas bacteria in 3 weeks and older biofilms showed increased resistance to these agents. The differences in the percentage of killed bacteria by the tested agents were greater in young biofilm, but whereas in older biofilms, these differences were noted less. This study results demonstrated that if young, non-matured biofilms are used to assess disinfecting agents' antibacterial efficacy, the results would be too optimistic. Most biofilm causing endodontic and periodontal infections are likely to be several weeks, months, or even years old at the treatment time. Therefore, when evaluating the antibacterial efficacy of endodontic irrigants, it's important to use mature biofilms. Bacteria in mature biofilms are more resistant to disinfectants than their planktonic counterparts because of the physical barrier of biofilm matrix, physiological state of biofilm bacteria (starved phase), as well as the existence of subpopulations known as "persisters."

Medium:

Jiang et al. 2017 ⁽⁶⁰⁾ stated that when the growth medium is not refreshed, nutrients become increasingly scarce, while concentrations of metabolic end-products and toxic waste products increase. This condition imposes stress on the bacteria, and one of the most important effects is a decrease in the biofilm cells'

metabolic activity. It's been shown that stressed or starved biofilms are less vulnerable to hostile conditions or antimicrobial treatment than non-stressed or non-starved biofilm. Limited nutrient refreshment seems to ends in a stressed biofilm, less vulnerable to treatment, and seems to reflect the treated root canal's environment rather than the primary infection. Therefore, whether or not to starve or stress the biofilm seems to be inspired by the condition that's being simulated.

Cruz et al. 2018 ⁽⁶¹⁾ compared the effectiveness of different nutrient media on biofilm growth of clinical strains of *Enterococci* and *Staphylococci*, including multi-drug resistant representatives. The broths used were Tryptic Soy (TS, BD), Mueller Hinton broth (MHB), Tryptic Soy supplemented with 1% glucose (TSG, ICN Biomedicals, Irvine, CA, USA), or 2% glucose (TS2G), Brain Heart Infusion (BHI, Sigma-Aldrich, St Louis, MO, USA) and Brain Heart Infusion supplemented with 1% glucose (BHIG). This study showed that tryptic soy broth supplemented with 1% glucose was an optimal medium for maximum biofilm growth of all strains tested.

Sodium Hypochlorite (NaOCl):

Reyhani et al., 2016 ⁽⁶²⁾ evaluated the effectiveness of different sodium hypochlorite (NaOCl) concentrations in reducing bacterial growth in *Enterococcus faecalis* biofilms in root canals. The root canals of maxillary central incisors of 104 subjects underwent chemomechanical debridement; 5.25% sodium hypochlorite solution

was used for 3 minutes in the root canals. The samples were then immersed in 1 mL of 17% EDTA for 3 minutes. Finally, the root canals were rinsed with phosphate-buffered saline (PBS) solution. After removing the smear layer, the samples were sterilized. Then *E. faecalis* biofilms formed within the root canals at 4-, 6-, and 10-week intervals were evaluated. Each group was divided into 4 subgroups in terms of the anti-bacterial treatment: group 1: 1% NaOCl solution; group 2: 2.5% NaOCl solution; group 3: 5.25% NaOCl solution; and group 4: PBS solution. After preparation of root canal filings, the counts of live bacteria were calculated using the classic counting method, i.e., colony-forming units (CFU). This study indicated that when using 1% NaOCl solution, bacterial count in 10-week-old biofilm was higher than in a 6-week-old biofilm, and 6-week-old biofilm had a higher count than that was in the 4-week-old biofilm. 1% NaOCl resulted in a decrease number of bacteria in all three biofilm stages compared with the PBS biofilm that acted as the control. Thus in comparison, 2.5% and 5.25% NaOCl completely inhibited the growth of living cells in all three stages of biofilm incubation (4, 6 and 10 week-old biofilms).

Vahid Zand et al., 2016 ⁽⁶³⁾ compared the antibacterial efficacy of 2.5% sodium hypochlorite gel and 2.5% and 5.25% sodium hypochlorite solutions on *Enterococcus faecalis* (*E. faecalis*) biofilm. Sixty extracted human single-rooted teeth were taken, and the canal was contaminated with *E. faecalis* and incubated for six weeks. Then the teeth were divided into 3 experimental groups and

1 control group (n=15). The study protocol in the experimental groups consisted of an injection of 5 mL of each irrigant into the root canals. Samples were collected from the root canal walls, and 1:10 serial dilutions were prepared and added to Muller Hinton Agar (MHA) plates and incubated at 37°C for 48 h. A classic colony counting technique was used for determining vital *E. faecalis* bacterial counts in MHA plates. The results of the study showed no significant difference between the antimicrobial effects of NaOCl irrigation solutions at 2.5% and 5.25% concentrations against *E. faecalis*. This study also showed that although 2.5% NaOCl gel was effective in killing *E. faecalis*, it exhibited significantly lower antimicrobial efficacy in comparison to 2.5% and 5.25% NaOCl irrigation solutions. The lower antimicrobial efficacy of NaOCl gel in comparison to NaOCl solution could probably be attributed to the viscosity of gel and its inability to penetrate the depth of dentinal tubules.

Maria Teresa Arias-Moliz et al., 2016 ⁽⁶⁴⁾ determined the influence of dentin powder on pH, concentration, and antimicrobial activity of NaOCl alone and in combination with Etidronic acid (HEBP). A five-day-old *Enterococcus faecalis* biofilms grown on the surface of dentin blocks were exposed to 1% and 2.5% NaOCl alone or mixed with 9% HEBP for 3 minutes in the presence and absence of dentin powder. The biofilm formed was measured using a confocal laser scanning microscope by the live/dead technique. The available free chlorine and pH of the solutions were also measured. The presence

of dentin powder reduced the free available chlorine and pH in all the irrigating solutions. In the presence of dentin powder, 1% NaOCl lost its entire antimicrobial activity. In contrast, the antimicrobial activity in 2.5% NaOCl and 1% NaOCl/HEBP groups was significantly reduced, whereas it was not affected in the 2.5% NaOCl/HEBP group.

Saifalarab A. Mohammed et al., 2017 ⁽⁶⁵⁾ investigated the effect of sodium hypochlorite concentration and needle extension on removing *E. faecalis* biofilm formed on a 3D printed model. Biofilms were grown for ten days in the apical 3mm. Irrigation was performed with 9 mL of either 5.25% or 2.5% NaOCl or water for the 60s using a needle inserted either at 2 or 3 mm from the canal terminus. Fluorescence microscopy imaging was done, and residual biofilm percentages were calculated using imaging software. Residual biofilm was found less when using 5.25% than with 2.5% NaOCl. The study results showed statistically significant biofilm removal when the needle was placed closer to the canal terminus. Also, as the concentration increased, a greater reduction of available chlorine and pH was noted.

Salma Al Nesser et al., 2019 ⁽⁶⁶⁾ in their systematic review, investigated the in vitro antibacterial effect of sodium hypochlorite gel on *E. faecalis* during root canal treatment. Results showed that there is currently insufficient reliable evidence about the antibacterial effect of sodium hypochlorite gel on *Enterococcus faecalis*. According to the robust studies included in this review,

sodium hypochlorite solution was more effective than its gel form against *E. faecalis*. Further studies are needed to consider which type, concentration, duration, and treatment protocols of sodium hypochlorite are most effective.

Ghassan Yared et al., 2020 ⁽⁶⁷⁾ compared the different irrigation regimens using sodium hypochlorite (NaOCl) with and without ultrasonic and sonic agitation and with and without intracanal heating of NaOCl and tested their disinfecting ability. Extracted human mandibular premolars were prepared, autoclaved and inoculated with *E. faecalis* for 28 days. The canals were then randomly assigned into eight groups (n=10) depending on the NaOCl irrigation protocol. Group - CONV: conventional Irrigation with syringe and needle; Group - END: NaOCl sonic agitation with EndoActivator; Group - EDD: NaOCl sonic agitation with EDDY; Group - PUI: NaOCl passive ultrasonic agitation; Group - H: intracanal heating of NaOCl; Groups EDD-H, END-H and PUI-H: NaOCl agitation with EDDY, EndoActivator, and passive ultrasound respectively, followed by intracanal heating of NaOCl. The canals were sampled before (S1), and after different irrigation regimens were tested (S2), the colony-forming units were counted. The groups with NaOCl intracanal heating reduced bacteria significantly more than the other group. In group H, five S2 samples were bacteria-free. In the groups with NaOCl ultrasonic and sonic agitation followed by NaOCl heating, all S2 samples were bacteria-free. Intracanal warming of NaOCl was more effective in killing

bacteria than conventional syringe irrigation and passive ultrasonic or sonic agitation of NaOCl. The present study concluded that intracanal warming of 5.25% NaOCl might be considered an alternative to conventional irrigation protocols with sonic or ultrasonic agitation. Ultrasonic or sonic agitation of 5.25% NaOCl followed by intracanal heating is very promising regarding removing the infection.

Ethylene diamine tetracetic acid (EDTA):

S. George and A. Kishen 2008 ⁽⁶⁸⁾ investigated the surface characteristics, antibacterial effect and biofilm-forming potential of *E. faecalis* cells sequentially exposed to EDTA and calcium hydroxide. Bacterial cells exposed to calcium hydroxide and EDTA were assayed for cell viability, membrane integrity, cell surface hydrophobicity and surface charge, while alteration in the surface topography of *E. faecalis* cells was examined using atomic force microscopy (AFM). The potential adherence of the bacteria to type I collagen was also examined to assess the biofilm-forming capacity of *E. faecalis* cells exposed to EDTA and calcium hydroxide. It was found that calcium hydroxide treatment reduced the viability of *E. faecalis*. Prior exposure to EDTA significantly reduced the antibacterial effect of calcium hydroxide ($P<0.05$). Calcium hydroxide treatment resulted in impaired cell wall morphology, observed as increased surface roughness and pore formation under AFM. However, these topographical changes induced by calcium hydroxide were significantly reduced in EDTA pre-treated cells

($P < 0.05$). Calcium hydroxide treatment caused a reduction in hydrophobicity and adherence of *E. faecalis* to type I collagen. These effects due to calcium hydroxide were also significantly altered in EDTA-pre-treated cells. The study concluded that the antibacterial and anti-adherence effect of calcium hydroxide was diminished by prior exposure of *E. faecalis* cells to EDTA.

P. Neelakantan et al., 2014 ⁽⁶⁹⁾ investigated the antibiofilm efficacy of three irrigation protocols (a mixture of NaOCl and Etidronic acid, NaOCl-EDTA and NaOCl-EDTA-NaOCl) activated by ultrasonics, diode laser or erbium: yttrium aluminum garnet laser (Er: YAG) laser on mature *E. faecalis* biofilms grown on root dentine in vitro. Root canals were instrumented using a rotary NiTi system. Samples were assigned into three experimental ($n = 80$) and one control ($n = 40$) group according to the irrigation protocol employed: group 1 - (NaOCl + Etidronic acid), 1: 1 mixture of 6% NaOCl and 18% Etidronic acid; group 2 - (NaOCl-EDTA), 3% NaOCl followed by 17% EDTA; group 3 - (NaOCl-EDTA-NaOCl), 3% NaOCl followed by 17% EDTA and final irrigation with 3% NaOCl. Based on the activation method, samples were further divided into four subgroups ($n = 20$): subgroup A - no activation; subgroup B - ultrasonic activation; group C - diode laser; group D - Er: YAG laser. Bacterial viability was assessed by using a confocal laser scanning microscope. Root dentine powder was used to determine the colony-forming units. The results showed that the maximum bacterial reduction was brought about by group 1 (NaOCl

+ Etidronic acid) and group 3 (NaOCl-EDTA-NaOCl). About the irrigation protocol, the use of a disinfecting agent such as NaOCl after the use of EDTA and continuous chelation brought about significantly higher bacterial reduction. This means that chelation allowed better penetration of NaOCl into the dentinal tubules facilitating bacterial reduction. The process of continuous chelation prevents the formation of a smear layer. Hence, it is possible that NaOCl was able to penetrate the dentinal tubules better in this situation. It may also be speculated that the continuous chelation protocol could penetrate better into the bacterial biofilm matrix and disrupt it, thereby allowing better contact of NaOCl with the bacteria. Thus it was concluded that the intracanal biofilm was destroyed in all experimental groups. NaOCl + Etidronic acid or NaOCl-EDTA-NaOCl resulted in better dentinal tubule disinfection than NaOCl - EDTA.

XP Endo-finisher:

Adham A. Azim et al., 2016 ⁽²⁾ determined the efficiency of 4 irrigation systems in eliminating bacteria in root canals, particularly in dentinal tubules. Roots of human mandibular and molar teeth were prepared with Endo-sequence to 25/04, autoclaved, and inoculated with *Enterococcus faecalis* for three weeks. After 21 days, canals were ~~then~~ randomly divided into four groups (n=15 teeth/group) and disinfected as follows (1) standard needle irrigation (SNI), (2) sonic agitation with EndoActivator (EA), (3) XP Endo finisher, or (4) Er: YAG laser (PIPS) (15 roots/group).

MTT assays were used to determine the bacterial reduction in the canal. To measure live/dead bacteria in the dentinal tubules (4 teeth/group), teeth were sectioned vertically and stained with LIVE/DEAD Back Light agent. A confocal laser scanning microscope (CLSM) was used to determine the ratio of dead/total bacteria in coronal, middle and apical 3rd of root dentin at various depths. This study showed that all four irrigation protocols caused a significant bacterial reduction in the canal, ranging from 89.6% to 98.2% ($P < .001$). XP Endo finisher had the greatest bacterial reduction than the other three techniques ($P < .05$). CLSM analysis showed that XP Endo finisher had the highest dead bacteria level in the coronal, middle, and apical segments at 50-mm depth. On the other hand, the greatest bacterial killing efficiency was shown by PIPS at the 150-mm depth in all three root segments. They concluded that XP Endo appears stronger than PIPS and EA in disinfecting the main root canal space and up to 50 mm deep into the dentinal tubules. PIPS appears to be more effective than XP Endo and EA in deeper disinfection of the dentinal tubules.

Pingping Bao et al., 2017 ⁽³⁷⁾ compared the effectiveness of the XP-endo Finisher (XPF), passive ultrasonic irrigation (PUI), and conventional needle irrigation (CNI) in biofilm removal using an infected tooth model with an artificial apical groove. Fifty-four extracted human single-rooted premolars were selected. Each tooth was vertically sectioned into two halves, with a groove made in the canal wall's apical segment. After growing mixed bacteria biofilm

for four weeks, the sectioned halves were reassembled, followed by instrumentation using Vortex Blue files to size 40/.06. According to the final irrigation protocol, the instrumented teeth were randomly assigned to 6 groups ($n = 8$). Three different irrigant agitation techniques (XPF, PUI, and CNI) were performed, each with either continuous irrigation or 3-step irrigation. A scanning electron microscopic examination was done to evaluate the amount of residual biofilm left inside and outside the groove. This study showed that biofilm's robust growth was observed in each canal of the controls after four weeks. XPF had the highest biofilm removal efficacy inside and outside the groove, followed by PUI and CNI. It was concluded that XPF 2 group that followed the 3-step protocol, showed anti-biofilm efficacy better than XPF1 group with continuous irrigation inside the groove ($P < .05$). Thus the XP-endo Finisher, as an irrigant agitation device, may help to remove biofilm from difficult-to-reach areas in the root canal system.

Maria Cristina CARVALHO et al., 2019 ⁽⁷⁰⁾ evaluated the effectiveness of XP-Endo Finisher (XPF) with XP-Endo Shaper (XPS) or Reciproc Blue (RB) files in bacterial load reduction in oval-shaped root canals during chemomechanical preparation (CMP) with 2.5% sodium hypochlorite (NaOCl) or 0.9% saline solution (NaCl). Eighty mandibular incisors with a single oval-shaped root canal were inoculated with *E. faecalis*. Teeth were randomly distributed into eight experimental groups ($n = 10$): G1- XPS, G2 - XPS + XPF, G3 - RB, and G4 - RB + XPF. CMP was performed with

NaCl or NaOCl. Colony-forming unit count assessed the bacterial load reduction before (S1) and after (S2) CMP. This study showed that irrespective of the irrigating solution used, all instruments used in the oval-shaped root canals effectively reduced bacterial load. RB was more efficient than XP-S when 0.9% NaCl was used as root canal irrigant, as its design favors more contact of the instrument with the root canal walls, dislodging the biofilms and removing them during irrigation. The supplementary approach of XP- F was found to increase the cleaning efficacy of XP-S and RB systems achieving the greatest reduction in the 2.5% NaOCl group. This emphasizes the need to use antimicrobial substances during CMP for microbial control.

Ebtissam Almadi et al., 2019 ⁽⁵²⁾ compared several irrigation protocols for helping irrigation penetration into the dentinal tubule. Fifty-five extracted single-rooted human mandibular premolar roots were instrumented to size 30 with a taper of 4%, autoclaved and then inoculated with *Enterococcus faecalis* for three weeks. Teeth were divided according to the disinfection protocol into five groups, three experimental (n=15) and two control groups (n=5), group A - Diode Laser, group B - XP Endo Finisher, group C - needle irrigation, also, two control groups, groups D (n= 5): canals irrigated with Saline and E (n=5), bacteria were not inoculated in the canals. The teeth were split into halves and then scanned using confocal laser scanning microscopy to measure the ratio of live vs. dead bacteria in the canals' dentinal tubules. There was no

significant difference among all the groups($p<0.05$) between the amount of live vs. dead bacteria. The Diode laser and XP Endo finisher showed more dead bacteria at all levels. It was concluded that the Sodium hypochlorite activation by Diode laser and XP Endo finisher improves the root canal's disinfection at all levels, especially at the apical part. Canals irrigated with XP Endo Finisher and Diode Laser showed a higher bacterial reduction of *E. faecalis* in dentinal tubules than standard needle irrigation.

Harakh Chand Baranwal et al., 2019 ⁽⁷¹⁾ in their review article, discussed the newer generation file (XP Endo®) from FKG. XP Endo Finisher (XPEF) file was introduced by FKG in 2015. It was suggested to be used at 800 rpm and 1.0 Newton Torque with irrigating solution after root canal preparation to size #25 or longer. The instrument's diameter is ISO #0.25, and its taper is equal to 0. XPEF-R® File was introduced in 2017; FKG completes the range of these instruments with one dedicated to retreatment: the XP-Endo Finisher-RTM (XPEF - R). The XPEF - R has a stronger core than XPEF and a diameter of ISO #0.30. The quality of cleaning and removing gutta-percha was superior.

Parnwad Sasanakulet al, 2019 ⁽⁷²⁾ evaluated and compared the effectiveness of different disinfection procedures in teeth with large root canals. Ninety-four extracted human mandibular premolars were taken. They were prepared to get 10 mm long samples with 0.8-mm wide, apical foramen, 92 samples were inoculated_with

Enterococcus faecalis for 21 days, and scanning electron microscopic examination (n = 2) was done to confirm biofilm formation. The 90 infected teeth were randomly distributed into 9 groups: Group 1- no intervention (initial), Group 2 - 1.5% NaOCl irrigation (1.5 N), Group 3 - 2.5% NaOCl irrigation (2.5 N), Group 4 - 1.5 N + intermittent passive ultrasonic irrigation (PUI), Group 5 - 1.5 N + intermittent canal brushing with Navitip FX (NFX), Group 6 - 1.5 N + intermittent XP-endo Finisher (XPF), Group 7 - 1.5 N + circumferential filing (CF), Group 8 - 1.5 N + 1-min Self adjusting File (SAF), and Group 9 - 1.5 N + mechanical instrumentation using #90–110 files (MI). For microbial analysis, root canal walls were shaved using a No.4 Peeso reamer and mean colony-forming units (CFUs) were counted. The mean CFUs in each treatment group were significantly lower than the initial group ($P < .05$). The mean CFUs in the 1.5 N group as the highest among the irrigation groups. The mean CFUs of the 1.5 N group as approximately 3.6- fold higher than the PUI group; 4- to 5-fold higher than the 2.5 N, CF, and SAF groups; and 22-, 36-, and 464-fold higher than that of the PF, NFX, and MI groups, respectively. The 2.5 N group and 1.5 N groups with adjunctive treatments, excluding the PUI group, had significantly lower mean CFUs than the 1.5 N group ($P < .05$). Using the large root canal model, the results indicated that root canal disinfection using 1.5% NaOCl irrigation was significantly improved using supplemental procedures. Adjunctive root canal brushing using NFX

was the most promising alternative for improving root canal disinfection without dentin removal.

Nidambur Vasudev Ballal et al., 2020 ⁽⁷³⁾ evaluated the effect of NaOCl irrigant activation using passive ultrasonic irrigation (PUI), F-file (Engineered Endodontics), XP-endo Finisher file (XPF) and needle irrigation on the reduction of intracanal bacteria from teeth undergoing root canal treatment. Asymptomatic apical periodontitis patients (n=80) were randomly assigned into four groups. Access cavities were prepared, the apical canal was enlarged to size 20, pre-treatment samples were taken (S1), and post-instrumentation samples (S2) were taken. NaOCl was then activated with the various irrigant activation systems and post-irrigation samples (S3) taken. The samples were processed, and colony-forming units (CFUs) of bacteria were determined. For all groups, there was a significant reduction in the CFUs from S1 to S2 ($P<0.05$) and from S2 to S3 ($P<0.05$). There was no significant difference between XPF and PUI techniques; both were associated with the greatest reduction in CFUs ($P=0.24$). However, both XPF and PUI were associated with significantly lower CFUs than F-file activation and needle irrigation ($P<0.05$). There was a significant reduction in CFUs in the F-file activation method compared to needle irrigation ($P=0.04$). The XP-endo Finisher file's enhanced effect may be attributed to its spoon-shaped structure during activation and NaOCl irrigation that created irrigant streaming powerful enough to detach the microbes from the biofilm of the root canal system.

Passive ultrasonic Irrigation:

Vander Sluis et al., 2006 ⁽⁷⁴⁾ evaluated the literature on passive ultrasonic irrigation (PUI) to describe the mechanism and its effects and evaluated whether PUI is more effective in cleaning the root canal than syringe irrigation. PUI can be an important adjunctive for cleaning the root canal system (RCS), and it removes more planktonic bacteria, organic tissue, and dentine debris from the root canal when compared with traditional syringe irrigation. It was found that PUI is more efficient in cleaning canals when compared to ultrasonic irrigation with simultaneous ultrasonic instrumentation. The diameter and the taper of the root canal were found to be important parameters in determining the efficacies of dentine debris removal. PUI appears to be a supplement treatment for cleaning the RCS, and PUI is more effective than syringe irrigation.

B. Bhuva et al., 2010 ⁽⁷⁵⁾ compared the efficacy of passive ultrasonic irrigation (PUI) with 1% sodium hypochlorite (NaOCl) with that of conventional syringe irrigation (CSI) with 1% NaOCl, on intraradicular *Enterococcus faecalis* biofilms in extracted single-rooted human teeth. Root canal was prepared, sectioned longitudinally and *E. faecalis* were grown on the walls of 48 standardized root halves. Following reapproximation, the roots were randomly assigned into four groups (n=12). The two experimental groups were treated with Group A - conventional syringe irrigation with 1% NaOCl solution and Group B - passive ultrasonic irrigation

with 1% NaoCl solution. Of the two control groups, the first Group C was treated with conventional syringe irrigation with sterile saline solution (SSS), while the second control Group D - received no irrigation. The root halves were prepared for scanning electron microscopy, and three images (700x) were taken of the 12 root halves in each of the four groups at coronal, middle and apical, using a standardized protocol. Results of this study showed no significant differences in the scores for remaining biofilm coverage between group A (CSI with 1% NaoCl) and group B (PUI with 1% NaoCl) at the three observed levels. However, there was a significant difference between both (groups A and B) and group C (CSI with SSS) ($P < 0.001$) at all three observed levels. Both CSI and PUI with NaoCl solution were completely effective in removing the *E. faecalis* biofilm from the root canal walls of extracted human teeth. CSI with SSS was only partially effective in removing the biofilm.

Grasiela Longhi Grundling et al., 2011 ⁽⁷⁶⁾ evaluated the effect of ultrasonic irrigation of sodium hypochlorite (NaoCl) and EDTA in root canals of bovine teeth infected with *Enterococcus faecalis* in vitro. Eighty-four bovine incisors teeth were prepared and inoculated with *E. faecalis* for 50 days to form a biofilm. Teeth were randomly distributed into four groups: the conventional syringe irrigation (CSI) with NaoCl + EDTA group; and the passive ultrasonic irrigation (PUI) with NaoCl + EDTA group; the control group, which received no treatment; the ultrasonic + distilled water

group. Microbiological tests and scanning electron microscopy (SEM) analysis were performed. There were no significant differences between the CSI group and the PUI group in any of the thirds observed in the canal wall. However, there were significant differences between these groups and the control group in all thirds. It has also been shown that there was a significant difference between the ultrasonic + distilled water group and the control group in the apical and middle thirds and between-group ultrasonic + distilled water group and groups using NaOCl in the cervical and middle thirds. The authors of this study concluded that ultrasonics could aid in root canal cleaning, but the irrigant carries out a prominent role in eliminating bacteria.

Juliane Maria Guerreiro-Tanomaru et al., 2015 ⁽³⁵⁾ evaluated the effectiveness of PUI in eliminating *Enterococcus faecalis* from root canals ex-vivo. Seventy-five extracted human single-rooted teeth were taken, root canal was prepared, specimens were inoculated with *E. faecalis* and incubated for 21 days. Teeth were randomly assigned into five groups (n=15), as follows: conventional needle irrigation (CNI) with saline solution (CNI+SS); CNI with 1% NaOCl (CNI+NaOCl); PUI with saline solution (PUI+SS); PUI with 1% NaOCl (PUI+NaOCl); and No irrigation (control). Microbiological samples were collected at three-time points: initial (21 days after inoculation), post-irrigation (immediately after irrigation), and final (7 days after irrigation). This study showed that regardless of the irrigation method (CNI or PUI), 1% NaOCl

was the most effective irrigant against *E. faecalis*. However, seven days after irrigation, an increase in the bacterial counts was observed in all groups, demonstrating the permanence of *E. faecalis* in the root canal system (RCS). They concluded that both PUI and CNI with 1% NaOCl contribute to disinfection but are unable to eliminate *E. faecalis* from the RCS.

Patinee Pladisai et al., 2016 ⁽⁷⁷⁾ compared the effectiveness of different disinfection protocols in reducing *Enterococcus faecalis* biofilm bacteria in teeth with large root canals. Fifty-five human mandibular premolars with large single root canals were taken, and 50 roots were inoculated with *E. faecalis* suspension and incubated for 21 days at 37°C. Four roots were examined using scanning electron microscopy to confirm biofilm formation. The remaining specimens were assigned into five experimental groups and a sterile control group: irrigation with normal saline, irrigation with 2.5% sodium hypochlorite (NaOCl) (IR-N), mechanical instrumentation using 60–90 size k files and 2.5% NaOCl (MI-N), irrigation with 2.5% NaOCl using intermittent passive ultrasonic irrigation (PUI-N), and no intervention (initial). After root canal disinfection, dentin specimens were collected for microbial analysis. Results of this study showed the lowest number of intracanal bacteria was recovered from the MI-N group, followed by the PUI-N and IR-N groups. Irrigation with the normal saline did not show a significant reduction compared with the initial group. However, there were significant differences between groups ($P < .01$). The remaining

bacteria in the PUI-N group was 4.5-fold lower than the IR-N group; however, it was 1862-fold higher than the MI-N group. To disinfect a large root canal in which irrigant access to the apical portion was initially available, chemomechanical preparation was the most reliable disinfection protocol. Using an antibacterial agent supplemented with PUI improved conventional irrigation's effectiveness; however, none of the non-invasive protocols was as effective as chemomechanical root canal preparation.

Kariem Elbatouty et al., 2017 ⁽⁷⁸⁾ evaluated the antibacterial effect of diode laser 980 nm, Endovac and passive ultrasonic irrigation ex vivo. One hundred and five maxillary central incisors were standardized to 15mm in length. Teeth were prepared using ProTaper Universal rotary nickel-titanium system till size # F4 then contaminated with *E. faecalis*. The irrigation protocol used was 2.5% sodium hypochlorite, followed by 17% EDTA. Teeth were assigned into five groups (n= 20) according to the irrigant activation method. Laser activated irrigation (LAI) group - Diode laser 980nm, apical negative pressure irrigation (API) group - EndoVac system, PUI group - passive ultrasonic activation. The positive control group, in which the irrigating solution was not activated and the negative control group in which the samples were not subjected to irrigation or activation. Residual bacteria were collected with a sterile paper point, plated onto BHI media and incubated (37 °C, 48 h) to determine the colony-forming units. The current study results showed a significant reduction in the mean of

colony-forming units among all groups. However, none of the activation methods was able to kill *E. faecalis* biofilm completely. The LAI group behaved most effectively among all groups. The laser group showed the lowest residual bacterial load. This can be attributed to the laser energy that tends to accelerate the movement of irrigating solution pushing it into complicated canal anatomy.

EndoActivator:

Cameron Townsend et al., 2009 ⁽¹⁹⁾ determined whether needle irrigation, EndoVac irrigation, EndoActivator, F-File, and sonic agitation are as effective as ultrasonic agitation in mechanically removing bacteria in a simulated 30⁰ canal. Plastic blocks used in this study were distributed into seven groups. The control (C) group with brain-heart infusion (BHI) broth (sterile) received only needle irrigation. The remaining groups were incubated with BHI inoculated with *Enterococcus faecalis*. Irrigation and agitation techniques used were ultrasonic, needle irrigation, EndoVac irrigation, EndoActivator, F-File, and sonic with sterile water as an irrigant in all treatments. Results of this study showed that none of the irrigation or agitation techniques eliminated all intracanal bacteria. However, ultrasonic agitation was not significantly different ($p > 0.05$) from the control. No significant difference ($p > 0.05$) was found between the ultrasonic agitation and the use of F-File, Sonic agitation, and EndoActivator. Ultrasonic agitation was significantly more effective in eliminating bacteria than EndoVac irrigation and needle irrigation.

Andrew Halford et al., 2012 ⁽⁵⁰⁾ explored the combination of microbubbles with sonic or ultrasonic agitation within root canal models to characterize the bubble dynamics generated within a simulated root canal and to assess the effect on biofilm bacteria grown in canals of extracted teeth. High-speed imaging was done to characterize the bubble dynamics generated in microbubble emulsion (ME) by sonic or ultrasonic agitation. Secondly, 5.25% NaOCl irrigation or ME was sonically or ultrasonically agitated in canals of extracted teeth with 7-day-grown *Enterococcus faecalis* biofilms. Dentinal shavings from canal walls were sampled at 1 mm and 3 mm from the apical terminus, and colony-forming units (CFUs) were determined. High-speed imaging demonstrated strongly oscillating and vaporizing bubbles generated within ME during ultrasonic but not in sonic agitation. Compared with CFU counts in controls, NaOCl-sonic and NaOCl-ultrasonic yielded significantly lower counts ($P < .05$) at both measurement levels. ME-sonic yielded significantly lower counts ($P = .002$) at 3 mm, whereas ME-ultrasonic yielded highly significantly lower counts ($P = .000$) at both measurement levels. At 3 mm, ME-ultrasonic yielded significantly lower CFU counts ($P = .000$) than ME-sonic, NaOCl sonic, and NaOCl-ultrasonic.

Joan Mathew et al., 2014 ⁽¹⁾ evaluated the antimicrobial effect and viability of *Enterococcus faecalis* biofilms using conventional irrigation, EndoActivator, diode laser irradiation and photon-initiated photoacoustic streaming (PIPS). One hundred thirty single-

rooted human mandibular premolars, standardized to a length of 20 mm, were prepared to F1. After sterilization, five teeth were randomly selected to check sterility before being inoculated with bacteria. One hundred twenty-five samples were infected with *E. faecalis* for 21 days and divided into five groups (n = 25). In Group 1- positive control (root canals were not subjected to any treatment). Root canal sampling was performed to determine the colony-forming unit count for 20 samples. Five samples were selected to visualize colonization pattern by confocal laser scanning microscopy at Level 1 - 4 mm from the apex and Level 2 - 1 mm from the apex. Samples in Groups 2-5, namely conventional needle irrigation, EndoActivator, diode laser and PIPS, were subjected to their respective disinfection procedures. Post disinfection sample evaluation criteria were followed for all groups as same as that for Group 1. A significant reduction in the CFU count was found for all the techniques tested compared with the positive group. Diode laser displayed the highest antibacterial efficacy whereas conventional needle irrigation showed the least. There was a statistically significant difference in bacteria's viability at Levels 1 and 2 between the groups. The diode laser's superior bactericidal effect in the present study could be attributed to its greater penetration depth to 1000 µm into the dentinal tubules than the chemical disinfectants' penetration which is limited to 100µm.

Huseyin Gurkan GUNEC et al., 2018 ⁽⁷⁹⁾ compared the effectiveness of different final irrigant agitation techniques in

removing *Enterococcus faecalis* biofilms from root canals. Eighty-five extracted single-rooted human maxillary incisors teeth were instrumented using the Revo-S system to a 40/06 size. Eighty teeth were inoculated with *Enterococcus faecalis* and incubated for seven days. The remaining five teeth served as the negative control (without inoculation). Five out of 80 teeth were randomly taken as a positive control group; one tooth of the positive control group was analyzed using a scanning electron microscope (SEM) for biofilm formation remaining four teeth were not subjected to any final irrigant agitation. The remaining 75 teeth were divided into five groups (n=15) and rinsed with 5% NaOCl - 17% EDTA - 5% NaOCl. Following each irrigant application, agitation was performed for 60 s (3×20-s sessions) for different agitation techniques. Group 1 – manual dynamic agitation, Group 2 - passive ultrasonic agitation (PUI), Group 3 - EndoActivator (EA) agitation, Group 4 – photon initiated photoacoustic streaming (PIPS) with the Er: YAG laser and Group 5 -conventional syringe irrigation (CSI). Colony-forming units (CFUs) were measured in positive control and test groups. There was significantly better elimination of *E. faecalis* in the experimental groups than in the positive control groups ($p < 0.001$). CSI and Manual–dynamic agitation showed no significant differences between the two groups. PUI, EA and PIPS are considerably effective in removing *E. faecalis* biofilms from root canals with no significant differences between them.

Eugenio Pedulla et al., 2019 ⁽⁸⁰⁾ evaluated the immediate and residual antimicrobial activity of cordless sonic or ultrasonic devices on *Enterococcus faecalis* infected teeth. One hundred forty extracted single-rooted teeth infected with *E faecalis* were divided as follows (N = 15): Group 1- conventional syringe irrigation (CSI) with 3% sodium hypochlorite (NaoCl) activated by ultrasonic device, Group 2 – (CSI +NaoCl) activated by cordless ultrasonic device, Group 3 - (CSI +NaoCl) activated by EndoActivator (EA), Group 4 - (CSI +NaoCl) without activation, Group 5 - CSI with sterile bi-distilled water (DW) and ultrasonic device, Group 6 – (CSI+DW) activated by EndoUltra, Group 7 - (CSI+DW) activated by EA, Group 8 - (CSI+DW) without activation. The remaining 20 teeth served as positive and negative controls. Root canal samples were taken before, after irrigation, and after 24 hours of re-incubation and colony-forming units were measured. The antimicrobial efficacy of groups 1 and 2 was higher than groups 3 and 4 ($P < .05$). No bacteria reduction was found in groups 5-8 ($P > .05$). After 24 hours, the regrowth of bacteria was not significantly different for all groups ($P > .05$). Mechanical effects, such as acoustic streaming and cavitation, are dominant factors in ultrasonic-activated irrigation. Moreover, ultrasonic activation could cause the decomposition of the molecule and degassing of the solution. Sonic activation is expected to be less effective than ultrasonic activation due to lower frequency, resulting in the less acoustic stream. It was concluded that ultrasound produced lower

CFU and turbidity after treatment and after re-incubation of 24 hours than sonic or no activation.

Karina Gonzalez Camara Fernandes et al., 2020 ⁽⁵³⁾ analyzed three irrigation systems' effectiveness namely EndoActivator (EA), passive ultrasonic irrigation (PUI), and Easy Clean (EC) in reducing *Enterococcus faecalis*, after instrumentation with the reciprocating system, through microbiological collection and culture method. Sixty extracted human mandibular premolars were taken, accessed, infected with *E. faecalis*, and incubated for 21 days and instrumented with Wave One Primary. Before instrumentation, Initial collections (S1) were taken to confirm the contamination. As per the final irrigation protocol (n = 15), the teeth were divided into four groups: Group 1- EA; Group 2 - PUI; Group 3 - EC; and Group 4 - control group irrigated with saline solution and without agitation. In the final irrigation, 17% ethylene amine tetra-acetic acid (EDTA) and 2.5% sodium hypochlorite (NaOCl) was used in both; agitation was performed in three cycles of 20 seconds each. After agitation of the irrigating solutions, S2 samples were taken to measure colony-forming units (CFU/mL). After instrumentation and agitation, the control group showed significantly higher CFU/mL than the other groups, which were similar to each other. The results showed that all groups significantly reduced the number of bacteria in the root canals, except for the control group. They concluded that EC, PUI, and EA systems showed microbial reduction in root canals contaminated with *E. faecalis*, different from the control group.

Scanning electron microscope (SEM):

A. Kishen et al., 2006 ⁽⁸¹⁾ examined the interaction between *E. faecalis* biofilm and root canal dentine substrate (1) By examining the shift in the chemical composition of the biofilm structure with time and (2) by studying the topography and ultrastructure of the biofilm and the dentine substrate. Teeth were divided into two groups. In group 1, teeth were incubated with *E. faecalis* for 2, 4, and 6 week intervals. Teeth were subjected to X-ray diffraction and Fourier transform infrared (FTIR) spectroscopic examination to determine biofilm's chemical composition. In group 2, teeth were incubated with *E. faecalis* for six weeks. The biofilm's topography and ultrastructure were examined using scanning electron microscopy (SEM), light and confocal laser scanning microscopy. SEM and FTIR also examined the sediments formed from the bacterial interaction on the dentine (in group 1). These experiments highlighted different stages in the interaction of *E. faecalis* with root canal dentine. Further, bacterial-induced apatite reprecipitation on mature biofilm was also observed. This ability of *E. faecalis* to form such calcified biofilm on root canal dentine may be a factor that contributes to their persistence after endodontic treatment.

Burcu Ozses Ozkaya et al., 2017 ⁽³⁹⁾ evaluated the antimicrobial efficacy of Er: YAG laser activation with photon-initiated photoacoustic streaming (PIPS), Nd: YAG laser disinfection, and conventional irrigation on *Enterococcus faecalis* biofilms using scanning electron microscopy (SEM). Biofilms were grown on 110

root halves and divided into the following: Groups 1 and 2 (saline and 1% NaOCl with the apical position of PIPS, resp.), Groups 3 and 4 (saline and 1% NaOCl with the coronal position of PIPS, resp.), Groups 5 and 6 (Nd: YAG laser after saline and 1% NaOCl irrigation, resp.) and Groups 7, 8, and 9 (conventional irrigation with 1% NaOCl, 6% NaOCl, and saline, resp.). SEM images of the apical, middle and coronal levels were examined using a scoring system. Between Group 1 and 2, score differences were insignificant at all levels in the remaining biofilm. Group 4 had significantly greater bacterial elimination than Group 3 at all levels. Differences in Nd: YAG laser irradiation between Groups 5 and 6 were insignificant. Groups 7 and 8 were insignificantly different, except at the middle level. The Saline group had a higher percentage of biofilms than the others. In this study, PIPS activation with NaOCl eliminates more *E. faecalis* biofilms in all root canals regardless of the fiber tip position. When applying PIPS at the canal entrance, NaOCl more effectively eliminated the biofilm than saline (i.e., Groups 4 and 3, resp.) at all root levels. This finding could be attributed to the enhanced bactericidal, dissolution, and cleaning effects of NaOCl when activated by laser energy.

Confocal laser scanning microscopy (CLSM):

Ronald Ordinola Zapata et al., 2008 ⁽⁸²⁾ evaluated the potential of confocal laser scanning microscopy (CLSM) to identify live and dead *Enterococcus faecalis* in infected dentin. Eight cylindrical dentin specimens were infected with *Enterococcus faecalis* in BHI

for 21 days. After the experimental period, the specimens were stained with fluorescein diacetate (FDA) and propidium iodide (PI) or acridine orange (0.01%) and analyzed by CLSM. Two non-infected dentin specimens were used as negative controls. CLSM analysis showed discrimination between viable (green) and dead (red) bacteria in infected dentinal tubules which could be observed after staining with FDA/PI. Acridine orange showed the metabolic activity of the *E. faecalis* cells inside the dentinal tubules by its red fluorescence. Thus the viability of bacteria in infected dentin can be effectively determined in situ by CLSM.

Benlee Yap et al.'s 2014 ⁽⁶⁵⁾ aimed to establish both axenic and multi-species biofilms containing *E. faecalis* on dentine *in vitro* and compared the resistance of such *E. faecalis* biofilms to a clinically relevant concentration of NaOCl. Biofilms were developed using *E. faecalis* alone (axenic) or *Fusobacterium nucleatum* and *Streptococcus sanguinis* on dentine discs. Following treatment with either saline or 0.9% sodium hypochlorite (NaOCl), the viability of *E. faecalis* was determined by serial plating, and qualitative analysis was performed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). This study showed that 0.9% NaOCl is highly effective against multi-species biofilm or *E. faecalis* grown alone ($P = 0.0005$ and $P = 0.001$, respectively).

Qiang Li et al., 2020 ⁽⁸³⁾ compared the efficiency of four final irrigation protocols in bacterial inhibition and smear layer removal in root canal systems. Thirty roots were inoculated with

Enterococcus faecalis, prepared with ProTaper Universal files. The teeth were disinfected by conventional syringe irrigation (CSI), sonic agitation using the EndoActivator (EA), passive ultrasonic irrigation (PUI), or an M3 Max file. Teeth without root canal preparation served as control. Teeth with root canal preparation but without final irrigation served as post-instrumentation baseline control. After irrigation, the teeth were sectioned into two halves. One-half of each tooth was examined to assess smear layer removal by scanning electron microscopy (SEM), and the other half was examined to evaluate the depth of bacterial survival in dentinal tubules by confocal laser scanning microscopy (CLSM). All the groups (PUI, EA, and M3 Max) showed better smear layer removal efficacy than the CSI group, which was not treated with activation. No significant difference was found among the PUI, EA, and M3 Max groups, which may be related to this study's limited sample size. CLSM results revealed that PUI had the greatest bacterial inhibition depth in the coronal, middle, and apical thirds of the canal (all $P < 0.05$ vs. other groups). According to SEM and CLSM evaluation, PUI appears to have the best infection controlling ability in root canal systems.

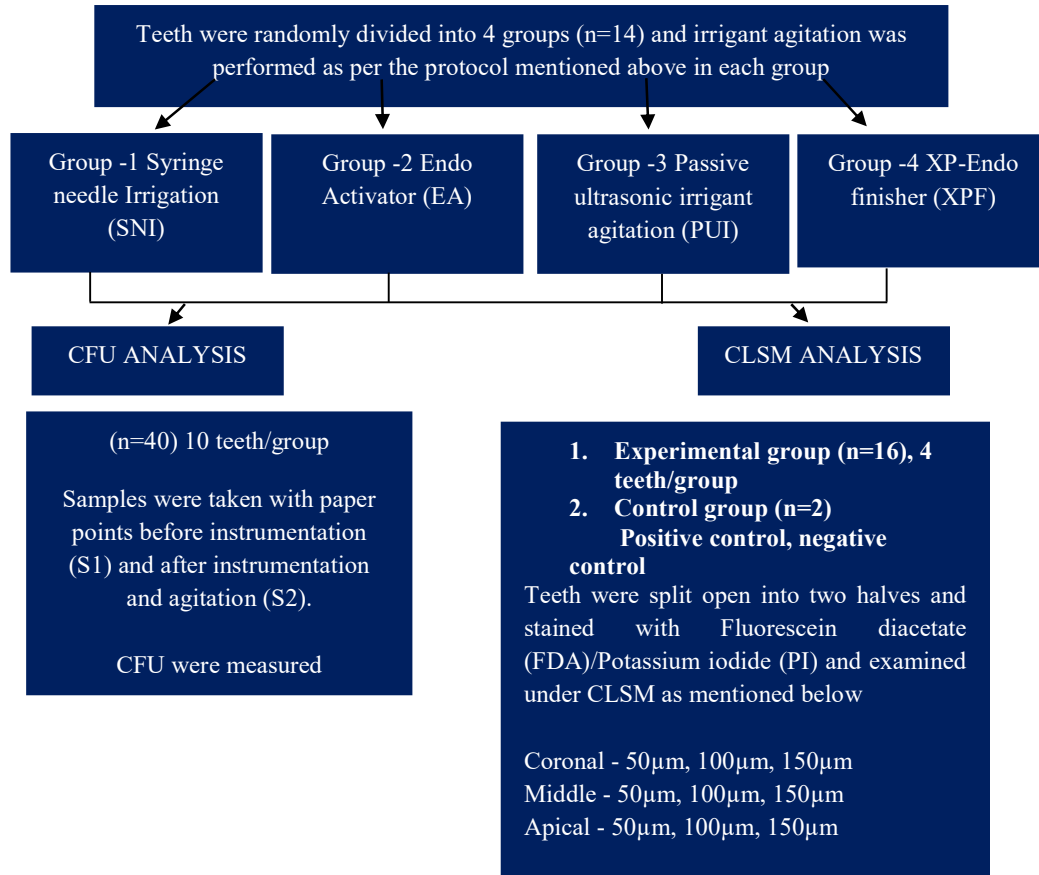
MATERIALS AND METHODS

MATERIALS: (Figure1)

1. Composite- flowable – 3M ESPE Filtek™ Z 350 XT – NA78024
2. Light cure unit – Coltene (Coltolux LED) – SN 160704300
3. MTwo rotary files- VDW, Munich, Germany, 296314
4. 3% Sodium Hypochlorite (NaOCl) – Chemident, India- 02318
5. 17% Ethylene diammine tetracetic acid (EDTA) –DE Smear EDTA -002
6. 2.5 ml syringes- Unolok 842026USJ2
7. Side vented needle- Prime RC Twents 2002-01
8. Ultrasonic Unit (PUI) -Coltene -Biosonic S1 SN 1508S1423C
9. Endoactivator – Dentsply maillefer CE 0086
- 10.XP Endo Finisher – FKG Dentaire – FK06
- 11.Contra angle hand piece- Waldent CA L180103204
- 12.X Smart Plus- Dentsply maillefer CE- 0459
- 13.Paper points – Sure endo CE 0197
- 14.Saline- Fresenius Kabi India pvt. Ltd. – 93N1204005
- 15.Spirit- HY CHEM 21B2760
- 16.Irrisafe tips – Acteon IRRI 20 21mm, F43807
- 17.K-files –MANI, R17D135500
- 18.Airotor handpiece – NSK, QM-T0308E

METHODOLOGY:





Study was approved by Institutional Human Ethics Committee
(Ref No: Sathyabama University/IHEC/Study no 042).

Study Design: *In-vitro*

Sample size calculation:

Mean 1=7.27, Mean 2=8.28, Mean 3=1.36, Mean 4=2.; SD 3.5⁽⁶⁹⁾,
(72), (53)

F tests - ANOVA: Fixed effects, omnibus, one-way

Analysis: A priori: Compute required sample size

Input:

Effect size f	= 0.8727844
α err prob	= 0.05
Power (1- β err prob)	= 0.95
Number of groups	= 4

Output: Non-°centrality parameter λ = 21.3290730

Critical F	= 3.0087866
Numerator df	= 3
Denominator df	= 24
Total sample size	= 28
Actual power	= 0.9596013

Collection of extracted human mandibular premolars:

After getting approval from the Institutional Human Ethical Committee from Sathyabama university extracted human mandibular premolars (figure 2) were collected from department of oral and maxillofacial surgery in Sathyabama dental college and

hospital and stored in distilled water at 4°C ⁽⁸⁴⁾ until used for the purpose of the study.

Inclusion criteria: Extracted human mandibular premolars with single root and single canal and presence of which was confirmed by radiographs taken in two different views ⁽⁸⁵⁾, (figure 3, 4) straight roots with curvature less than 5 degrees measured using Schneider's technique, intact and fully formed apices were included in the study.

Exclusion criteria: Teeth with calcified root canal, root resorption, immature root apex, canal with bucco-lingual: mesio-distal diameter > 2.5:1 when measured at 5mm from root apex, canal curvature greater than 5 degrees (figure 5) and previously endodontically treated teeth were excluded from the study. ⁽⁸⁶⁾



Figure 1: Armamentarium



Figure 2: Extracted Human Mandibular Premolars

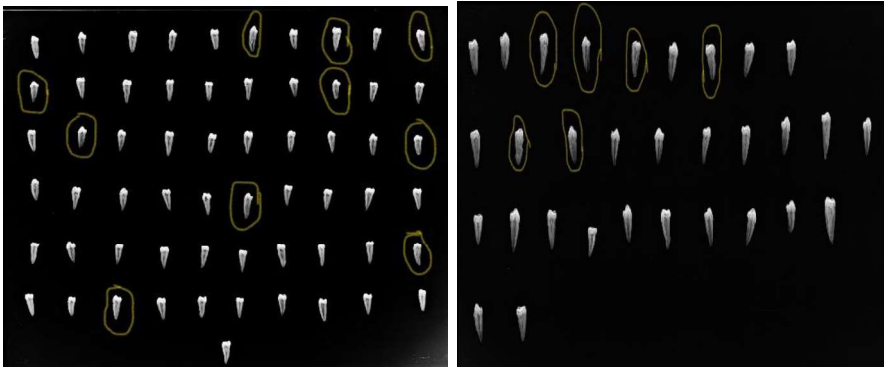


Figure 3: Radiograph taken in mesio-distal view
(Yellow markings indicates excluded teeth)

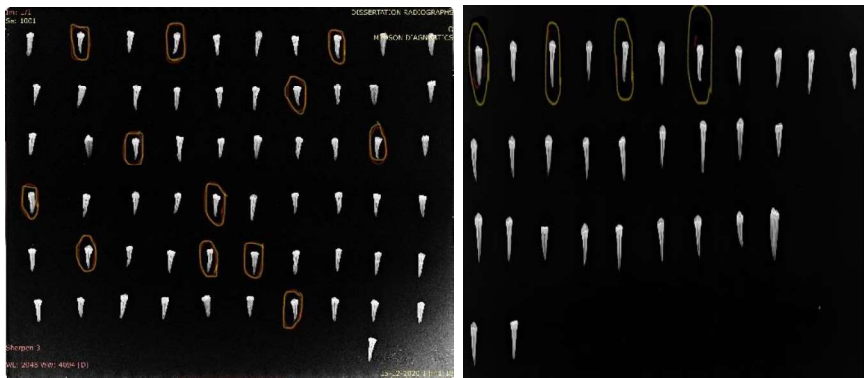


Figure 4: Radiograph taken in bucco-lingual view
(Yellow markings indicates excluded teeth)

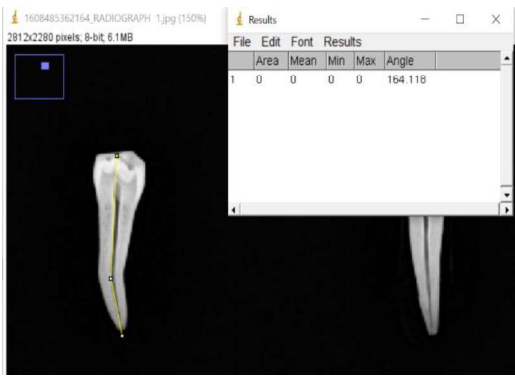


Figure 5: Diagrammatic representation of Schneider's method

One hundred and fourteen teeth were collected out of which 14 were excluded due to ratio of bucco-lingual: mesio-distal diameter greater 2.5:1 when measured at 5mm from root apex, 38 teeth were excluded due to curvature greater than 5 degrees, and two teeth were excluded due to internal root resorption.

Specimen preparation:

Canals were accessed using high speed handpiece (NSK, Japan) and diamond bur with a rounded cutting end (figure 6). The tooth length was measured by inserting a #10 stainless steel (SS) K-file (MANI, INC) into the canal until the file's tip was just visible at the apical foramen under magnification with loupes 2.5x. The cuspal reduction (figure 7) until each tooth measured 19mm in length, and the working length (WL) was standardized to 18 mm ⁽⁴¹⁾.

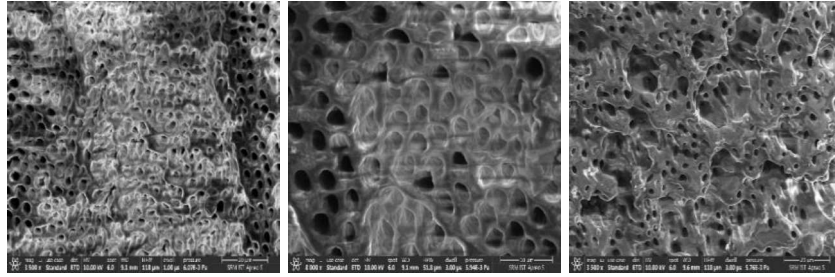


Figure 6: Preparation of access cavity



Figure 7: Cuspal reduction done to standardize tooth length

To standardize the apical geometry, the canal was instrumented to the working length (WL) with a size #15 SS K-file (MANI) followed by MTwo (VDW, Munich, Germany) #10/.04%, #15/.05%, #20/.06% rotary NiTi instruments. Only teeth where debris removal was visible in the apical 4-mm portion of the #20/6% file were included, all the teeth included in this study showed debris removal, so none of the them were excluded based on the present criteria.⁽³⁷⁾ During instrumentation, the canal was rinsed with 2 ml of 3% NaOCl (Chemident, India) with a flow rate of 2 ml/min (at room temperature) with a 2.5ml syringe (UNOLOK) using 30 guage needle (RC prime Twents, India) before each file and after the last file. A standard volume of 8 mL of 3% NaOCl was used per canal. On completion of canal preparation till #20/.06%, the canal was irrigated with 5 mL of 17% EDTA for 1 min, followed by 5ml of distilled water and a final rinse with 5ml of 3% NaOCl using a 30-gauge side vented needle for smear layer removal.⁽⁸⁴⁾ Teeth were autoclaved at 121°C for 15 min just before use. Three samples were randomly taken to ensure smear layer removal (figure 8) before bacterial inoculation by SEM at 5000x and 8000x magnification; sterility (figure 9) was assessed by measuring colony-forming units (CFU).⁽⁸⁷⁾



a. Coronal third b. Middle third c. Apical third

Figure 8: SEM images shows complete removal of smear layer at coronal, middle and apical third at 5000x and 8000x magnification.



Figure 9: Assessment of sterility of tooth samples using CFU. Culture plates shows no growth after 24 hours of incubation

Inoculation of specimens with *Enterococcus faecalis* for biofilm formation:

E. faecalis ATCC 29212 culture (24-hour culture) was prepared in brain heart infusion (BHI) broth, and the turbidity was adjusted to 0.5 McFarland standards (figure 10) to obtain a cell density of 1.5×10^8 cells/ml. Each canal was then filled to the orifice level with 10 μ l of *E. faecalis* suspension using sterile 1-mL insulin syringes with a 30-gauge needle. Then sterile K-type file #15 was

used to carry the bacterial suspension to the entire root canal length. ^{(2), (3)} Teeth samples were then placed in a 15-mL tube containing 10 mL BHIB. The inoculated teeth were then incubated at 37°C for 28 days in a shaking incubator (figure 11) (ORBITEK, Scigenics Biotech). The culture medium was replaced with a fresh medium every 3rd day. ⁽²⁾



Figure 10: Turbidity adjusted to 0.5 McFarland standards



Figure 11: *E. faecalis* inoculated teeth placed in the shaker

Confirmation of biofilm formation:

After 28 days of incubation, three teeth were randomly selected to confirm the biofilm formation using Scanning Electron Microscopy (SEM). Teeth were fixed in 10% buffered formalin. ⁽⁸⁶⁾ A diamond disc was used to cut a groove along the tooth's long axis stopping short of the canal lumen. A chisel was then used to split the tooth open into two pieces and then dried in ascending ethanol concentrations. They were then dehydrated to their critical point in

CO₂ and sputter-coated with gold under vacuum and viewed under SEM. ⁽⁸⁸⁾

Root canal instrumentation and irrigant agitation procedures:

After 28 days, teeth were removed from the inoculation tubes, and the root apices were sealed with flowable composite resin (3M ESPE Filtek™) in a clean environment laminar flow cabinet (figure 12) to prevent sample contamination and also to prevent leakage of canal contents. ⁽²⁾ Root canals were then instrumented using XSmart Plus Endomotor (Dentsply) and MTwo rotary files (figure 13) in the following order #25.06%, #30.05%, #35.04%, and the canal was rinsed with 2 ml of 3% NaOCl with the flow rate of 2ml/min (at room temperature) before each file and after the last file with a total volume of 8 ml of 3% NaOCl per canal. ⁽³⁷⁾

The teeth were then randomly divided into four groups (n = 10 teeth per group). In each group, the canal was rinsed with 2 ml 17% EDTA (DE Smear) for 1 minute using a 30-gauge side-vented needle, followed by 6 ml of 3% NaOCl for three cycles of 40 seconds with a total irrigation time of 2 minutes. Each cycle was subdivided into 20-seconds of irrigant agitation (agitation device according to respective groups) and 20-seconds refreshment.



Figure 12: Root canal apices sealed using composite resin



Figure 13: Root canal instrumented using MTwo Rotary files

Group 1: Syringe Needle Irrigation (SNI) - In this group, the canal was irrigated with 2 mL 17% EDTA for 1 minute using a 30-gauge side-vented needle placed within 2mm from the working length adjusted by a silicon stopper and moved in a vertical motion to avoid the needle being locked in the canal. After irrigation with 17% EDTA, canal was rinsed with 5ml of distilled water to flush out EDTA. Canal was then irrigated with 6ml of 3% NaOCl. Irrigation with 3% NaOCl in this group consists of 3 cycles of 40 seconds each, with a total duration of 2 minutes. In each cycle, the canal was irrigated with 2ml of 3% NaOCl for 20 seconds (figure 14) followed by 20 seconds of no irrigation. The same process was repeated for three cycles.



Figure 14: Root canal irrigated with 30-gauge side vented needle



Figure 15: Root canal irrigant agitated with EndoActivator tip

Group 2: EndoActivator (EA) – In this group, the canal was irrigated with 2ml of 17% EDTA for one minute and agitated with EndoActivator #25/.04% (Dentsply maillefer CE) at 10,000 cycles per minute (CPM) and EDTA was flushed out with 5ml of distilled Water. Canal was then irrigated with 6 ml of 3% NaOCl at a flow rate of 2 ml/20 seconds with the following cycles. Each cycle consists of 20 seconds of 2ml of 3% NaOCl irrigation (2 ml/20 sec) followed by 20 seconds of irrigant agitation with EndoActivator #25/4% at 10,000 CPM placed 2 mm short of the working length (figure 15). This process was repeated for three cycles.

Group 3: Passive ultrasonic irrigation (PUI) – In this group, the canal was rinsed with 2 ml of 17% EDTA by using a 30-gauge side-vented needle and agitated with PUI for 1 minute using Irrisafe tip # 20 (Acteon IRRI 20, 21mm) at a power setting of 3, (Coltene –Biosonic scaler unit, piezoelectric) and EDTA was flushed out with 5ml of distilled water then canal was irrigated with 6 ml of 3% NaOCl at a flow rate of 2 ml/20 secs for three cycles, each cycle for 40 seconds. Each cycle consists of 20 seconds of 2 ml 3% NaOCl irrigation (2 ml/20 sec) followed by 20 seconds of irrigant agitation (figure 16) with Irrisafe tip # 20 at a power setting of 3. The tip was placed 2 mm short from the WL, and this process was repeated for three cycles.

Group 4: XP Endo Finisher (XPF) – In this group, the working length was set in XPF using the plastic tube and rubber stop. The canal was flushed with 2 ml of 17% EDTA for 1 minute using a 30-gauge side-vented needle and agitated with XPF by inserting it slightly into the canal, then starting the motor and XPF (FKG Dentaire) was slowly threaded into the canal. It was operated at a speed of 800 rpm and a torque of 1 Ncm according to the manufacturer's instructions ⁽²⁶⁾ EDTA was flushed out with 5ml of distilled Water. Canal was then rinsed with 6 ml of 3% NaOCl at a flow rate of 2 ml/20 secs for three cycles. Each cycle consists of 20 seconds of 2ml of 3% NaOCl irrigation (2 ml/20 sec) followed by 20 seconds of irrigant agitation with XPF using slow and gentle 7-8 mm longitudinal movements ⁽²⁶⁾ to contact the entire length of the canal, and small multidirectional movements were made without removing the file from the canal (figure 17). The same process was repeated for three cycles.



Figure 16: Root canal irrigant agitated using passive ultrasonic (Irrisafe) tip

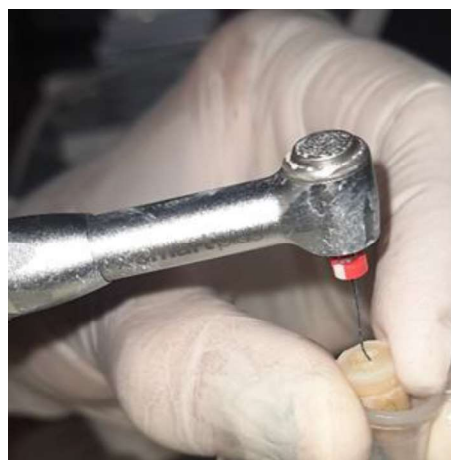


Figure 17: Root canal irrigant agitated with XP Endo finisher

***E. faecalis* sampling from the main canal to assess colony forming units:**

After the 28 days of bacterial incubation with *E. faecalis* samples were obtained from each canal before instrumentation (S1) and; after instrumentation and irrigation agitation (S2) to detect the viable bacterial count in the main canal.

S1 Sample - The root canal was first gently rinsed with 1 ml of sterile saline solution to remove the planktonic cells. Samples were collected using 3–5 paper points (#20/.06%) sequentially placed to the WL after agitating the fluid in the canal with 20/6% gutta-percha (figure 18) (GP) (MTwo, VDW). ⁽⁸⁸⁾ Each paper point was left to soak in the canal for one minute (figure 19). Paper points were then transferred to Eppendorf tubes containing 1 ml of sterile 0.85% saline solution and immediately processed. ⁽⁸⁹⁾



Figure 18: Agitating the fluid in the canal with gutta-percha



Figure 19: *E. faecalis* sampling with paper points

S2 Sample - Root canal was flooded with 10% sodium thiosulfate to neutralize the effect of sodium hypochlorite. Sampled by agitating the fluid in the canal with a sterile #35.04% GP point for about 25 strokes and then the contents were absorbed with 3-5 sterile paper points (#35/0.4%) until the canal became dry. ⁽⁸⁸⁾

Paper points used for taking S1 and S2 samples were transferred to Eppendorf tubes (figure 20) containing 1 ml of sterile saline and were immediately processed. Sample processing involved agitation of tubes containing the paper points in saline with the vortex mixer for 1 minute. 10 µl of the processed solution was immediately inoculated onto Mueller Hinton agar plate (spread plate method) and incubated for 24 hours and colony-forming units (CFUs) were counted using a digital colony counter.

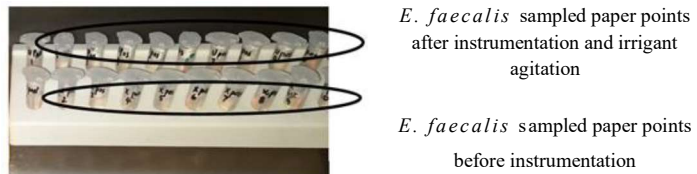


Figure 20: *E. faecalis* sampled paper points in Eppendorf tubes

CLSM analysis to evaluate bacterial reduction within the dentinal tubules:

Eighteen intact human mandibular premolars were used for this part of the study. Experimental groups (n=16) with 4 teeth/group and two teeth served as controls. After the 28-days of bacterial incubation, one tooth was autoclaved and used as a

negative control. The other tooth did not receive canal disinfection and was used as a positive control. ⁽²⁾

Roots were split vertically into two halves. Briefly, a groove was cut along the long axis of the tooth without penetrating the root canal system. Using a chisel, the root was split open into two pieces (figure 21). ⁽⁵²⁾ All samples were visually inspected to select two samples from each group with comparable canal dimensions for CLSM analysis. The specimens were then dyed using Propidium Iodide (PI) and Fluorescein Diacetate (FDA) to distinguish dead and live bacterial cells at different depths within the dentinal tubules. Bacteria with damaged cell membranes will be colored red, while cells with intact membranes will be colored green. ⁽²⁹⁾ Three random locations on the root were scanned at each part, coronal, middle, and apical (total of 9 images/tooth). For each image, 3 measurements were taken, representing 3 different depth levels: 50 μm , 100 μm , and 150 μm deep into the dentinal tubules.



Figure 21: Vertically split samples for CLSM analysis

STATISTICAL ANALYSIS

Statistical analysis was done using SPSS version 23. Data entry was done using Microsoft Excel 2016. For CFU, the approximate bacterial reduction was calculated for each tooth using the following formula:

$$\text{Bacterial reduction percentage} = \frac{S_1 - S_2}{S_1} \times 100\%$$

Wilcoxon-signed rank test was performed to determine the difference in CFU before instrumentation and after instrumentation and irrigant agitation within in the group in the main canal. Because the data were not normally distributed, Kruskal-Wallis test was used to test the overall difference among groups. Incidence of negative cultures was also compared between the four groups (SNI, EA, PUI, and XPF) by using the two-tailed Fisher exact test.

For CLSM analysis, the percentage of red to red-green combined was calculated for each tooth in all groups:

$$\text{Percent of bacterial reduction} = \frac{\text{intensity of red}}{\text{intensity of red} + \text{intensity of green}} \times 100$$

Three factors of analysis of variance were fit by using treatment groups (SNI, EA, PUI, and XPF), locations (coronal, mid-root, or apical), and depth points (50 µm, 100 µm, or 150 µm) as

factors. Shapiro-wilks test was applied to check the normality of the data and was found that the data was not normally distributed, hence non parametric tests such as Kruskal-wallis test and Mann-Whitney U test were applied to see the mean difference between and within the groups. P value < 0.05 was considered to be significant throughout the study. Descriptive statistics was given by mean, minimum, maximum and graphs.

RESULTS

Confirmation of biofilm formation by SEM:

Root canals showed to be infected by *E. faecalis* after 28 days of incubation. The samples showed *E. faecalis* biofilm on canal wall (figure 22).

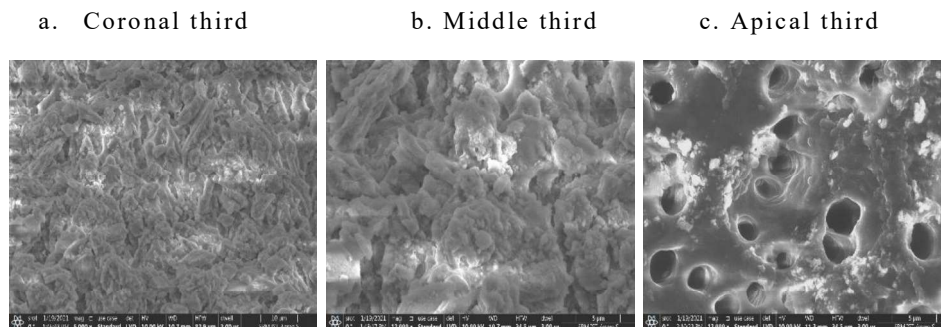


Figure 22: A thick, matured *Enterococcus faecalis* biofilm was formed after 28 days of incubation

Efficiency of Bacterial Elimination in Canal Space:

The CFU method measured the cultivable bacteria from the sample collection before and after root canal instrumentation and irrigant agitation. Results and images are displayed in Table -1, 2 and figure 23 respectively. All the groups showed statistically significant reduction in CFU after instrumentation and irrigant agitation as compared to pre-instrumentation with the P value of 0.05 in SNI, 0.006 in EA, 0.04 in PUI, and 0.03 in XPF as shown in table 1. The mean percentage reduction of bacteria ranged between 56.40% - 99.81% (SNI-56.40%, EA-96.99%, PUI-98.07%, and 99.81-XPF). Comparison of mean reduction in CFU between the groups did not show statistically significant difference with p value < 0.67.

WILCOXON - SIGNED RANK TEST:

Statistics									
	Pre-instrumentation SNI (Group-1)	Post-instrumentation SNI (Group-1)	Pre-instrumentation EA (Group-2)	Post-instrumentation EA (Group-2)	Pre-instrumentation PUI (Group-3)	Post-instrumentation PUI (Group-3)	Pre-instrumentation XP (Group-4)	Post-instrumentation XP (Group-4)	
Mean	1,05,650.00	53,200.00	79,810.00	1,020.00	86,660.00	1,700.00	1,43,500.00	290.00	
Median	1,01,050.00	20,800.00	80,500.00	.00	91,000.00	300.00	1,76,000.00	100.00	
Std. Deviation	34941.451	62800.619	45296.123	3155.524	26156.121	4330.768	57773.293	504.315	
p-value	0.05		0.006		0.04		0.03		
Range	145600	150000	153000	10000	79200	14000	172800	1600	

Table 1: All groups shows statistically significant reduction in CFU after instrumentation and irrigant agitation (S2) as compared to pre-instrumentation (S1) with p value of equal to or less than 0.05.

Kruskal-wallis test:

Statistics				
	Syringe Needle Irrigation (SNI) (Group-1)	EndoActivator (EA) (Group-2)	Passive Ultrasonic Irrigation (PUI) (Group-3)	XP- Endo finisher XPF (Group-4)
Mean difference	56.40	96.99	98.07	99.81
Median	89.80	100.00	99.15	99.94
Range	147	20	13	1
P- VALUE	0.67			

Table 2: Efficiency of bacterial elimination in the main canal by different disinfection methods. There were no significant mean differences between any of the groups with p value < 0.67.

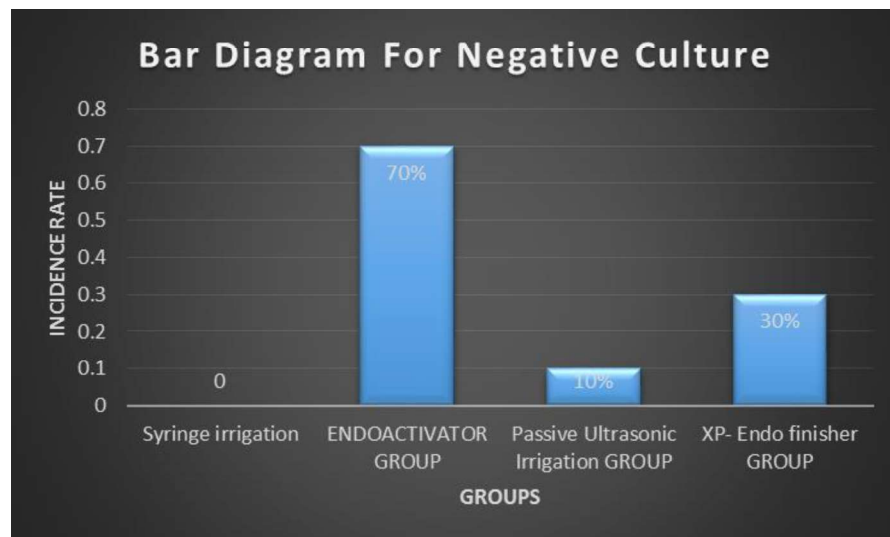
Incidence of negative cultures was compared between the 4 groups (SNI -0, EA-70%, PUI-10%, and XPF-30%) by using the two-tailed Fisher exact test (table 3), (graph 1).

Groups	SNI (Group-1)	EA (Group-2)	PUI (Group-3)	XPF (Group-4)
Incidence of negative cultures percentage	0	7/10x100=70%	1/10x100=10%	3/10x100=30%
p-value	(0.03)			

Table 3: Table showing incidence of negative cultures. There was significant difference between the groups with P-Value of 0.03

	Mean-pre instrumentation	Mean- Post instrumentation	Range	Mean- %bacterial reduction	Median- %bacterial reduction
SNI (Group-1)	1.05×10^5	5.3×10^4	147	56.40	89.90
EA (Group-2)	7.9×10^4	1.0×10^3	20	96.99	100.0
PUI (Group-3)	8.6×10^4	1.7×10^3	13	98.07	99.15
XPF (Group-4)	1.4×10^5	2.9×10^2	1	99.81	99.94

Table 4: Counts of *E. faecalis* CFUs before instrumentation (S1) and after instrumentation with irrigant agitation (S2)



Graph 1: Bar graph showing significant difference between incidence rate of different group with p-value <0.05(0.03)

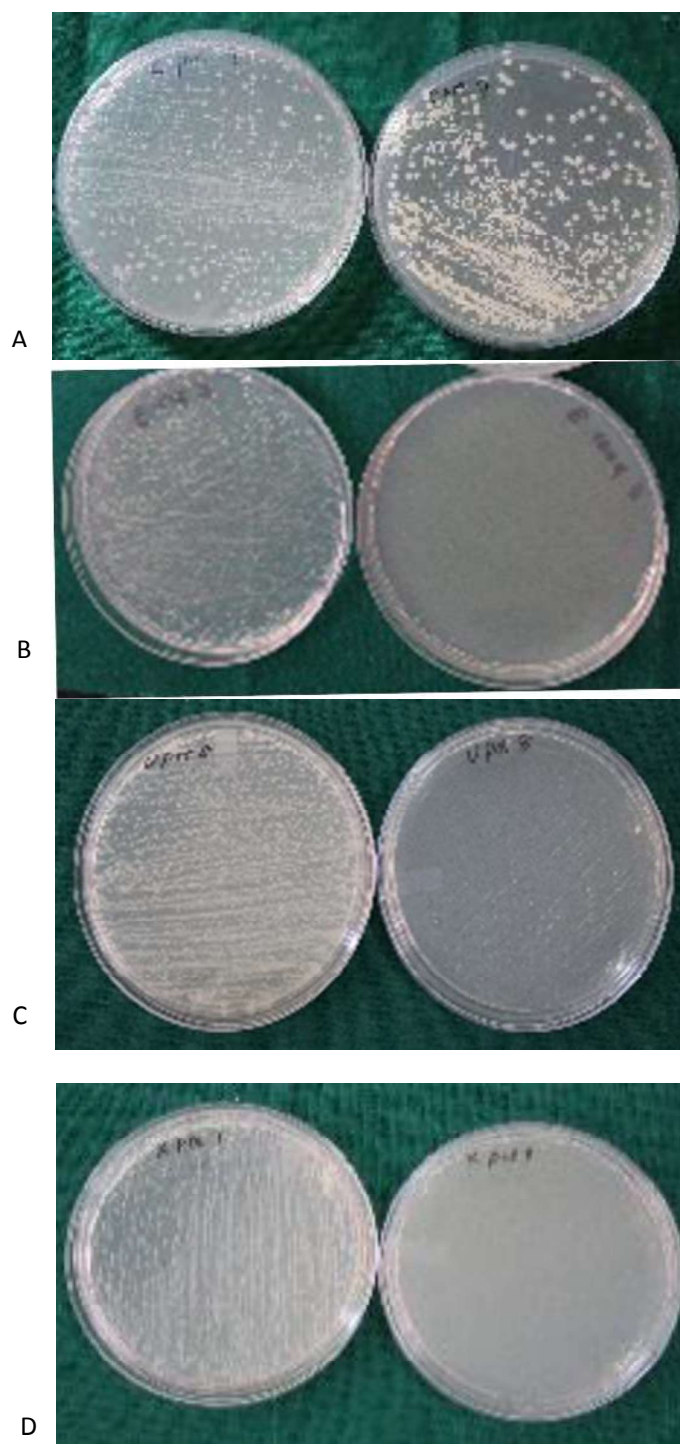


Figure 23: Culture plates showing bacterial growth before instrumentation and after instrumentation and agitation. (left culture plate in each image is representative of bacterial growth - pre-instrumentation and agitation and right image is representative

of bacterial growth - post instrumentation and agitation) a. Conventional syringe needle irrigation group, b. Endoactivator group, c. passive ultrasonic irrigation group, and d. XP Endo-Finisher group.

Efficiency of Killing Bacteria within Dentinal Tubules:

CLSM analysis was done to detect live versus dead bacteria in the dentinal tubules after staining. All the groups showed dead bacteria at all the thirds of root and at all depths tested. The percentage of dead bacteria (red) over total bacteria was calculated along the vertical lines placed on each image as described in (figure 24). The results from analyzing those images are presented in table 5.

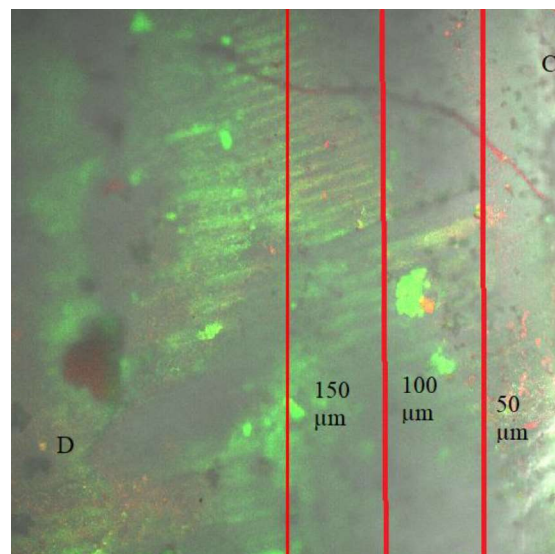


Figure 24: Method of confocal imaging analysis of live/dead bacteria in dentinal tubules. Representative image after image stacking showing the canal side C and dentin side D, revealing dead bacteria (red) and live bacteria (green). At different depth planes within the dentinal tubules, measurements were taken at 50, 100, and 150 mm deep into the dentinal tubules.

In Figure 25, the negative control showed no live bacteria in the dentinal tubules, whereas dead bacteria were present. In contrast, the positive control had many live bacteria in the dentinal tubules. As shown in 26 representative images depict live/dead bacteria in the dentinal tubules of different segments (coronal, middle, and apical) in roots treated by the 4 disinfection procedures.

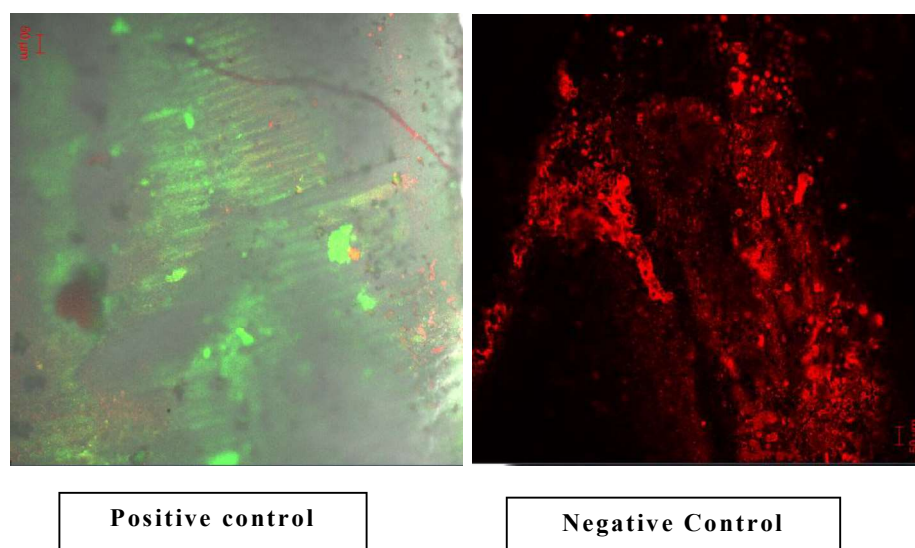


Figure 25: CLSM analysis of positive and negative control group. Positive and negative control sample images. Red indicates dead bacteria and green indicates live bacteria in dentinal tubules.

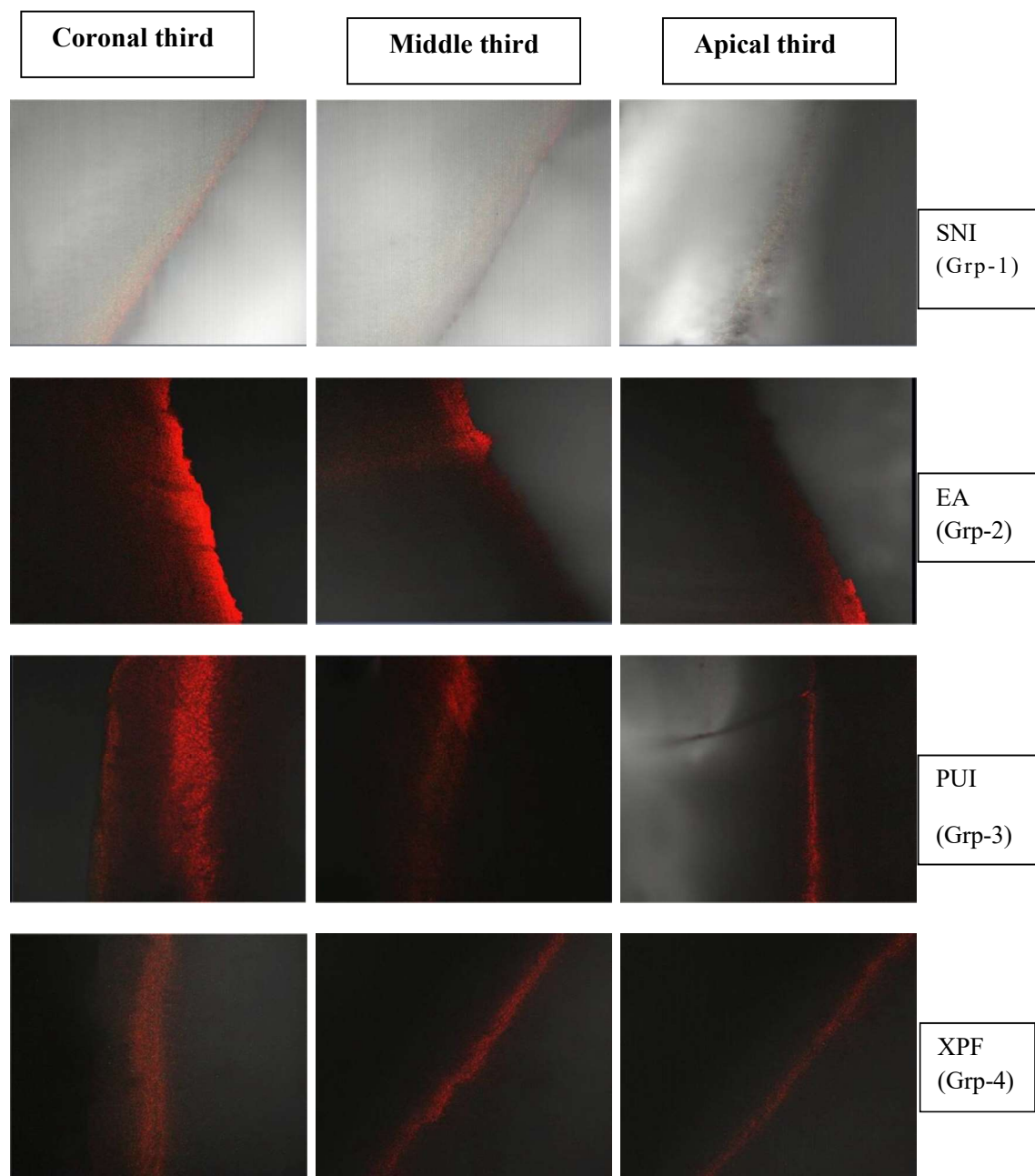


Figure 26: CLSM analysis of live/dead bacteria in dentinal tubules. Representative stacked images from coronal, middle, and apical thirds of the root showing dentinal tubules in samples treated by different irrigation methods: SNI, EA, PUI and XPF

Kruskal-wallis test:

		Descriptive Analysis				
Grouping variables		N	Mean	Minimum	Maximum	P-VALUE
SNI (Group-1)	coronal 50	1	.522617	.5226	.5226	0.317
	middle 50	1	.451150	.4511	.4511	0.517
	apical 50	1	.411883	.4119	.4119	0.34
	coronal 100	1	.362267	.3623	.3623	0.57
	middle 100	1	.289650	.2896	.2896	0.54
	apical 100	1	.245183	.2452	.2452	0.34
	coronal 150	1	.209533	.2095	.2095	0.24
	middle 150	1	.185050	.1850	.1850	0.6
	apical 150	1	.092418	.0924	.0924	0.24
	Total	9	.307750	.0924	.5226	0.42
EA (Group-2)	coronal 50	1	.938117	.9381	.9381	0.52
	middle 50	1	.903500	.9035	.9035	0.15
	apical 50	1	.735300	.7353	.7353	0.34
	coronal 100	1	.835840	.8358	.8358	0.34
	middle 100	1	.741500	.7415	.7415	0.21
	apical 100	1	.508700	.5087	.5087	0.13
	coronal 150	1	.664667	.6647	.6647	0.45
	middle 150	1	.491667	.4917	.4917	0.32
	apical 150	1	.295650	.2957	.2957	0.9
	Total	9	.679438	.2957	.9381	0.89
PUI (Group-3)	coronal 50	1	.891183	.8912	.8912	0.78
	middle 50	1	.802050	.8020	.8020	0.7
	apical 50	1	.647250	.6473	.6473	0.56
	coronal 100	1	.817783	.8178	.8178	0.98

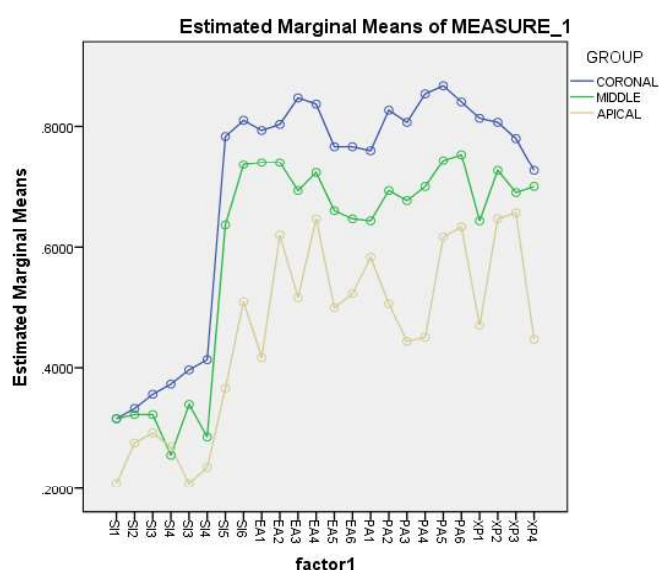
	middle 100	1	.699767	.6998	.6998	0.56
	apical 100	1	.501667	.5017	.5017	0.78
	coronal 150	1	.674983	.6750	.6750	0.34
	middle 150	1	.508117	.5081	.5081	0.67
	apical 150	1	.354150	.3541	.3541	0.77
	Total	9	.655217	.3541	.8912	0.66
XPF (Group-4)	coronal 50	1	.928100	.9281	.9281	0.88
	middle 50	1	.872550	.8726	.8726	0.99
	apical 50	1	.717050	.7171	.7171	0.77
	coronal 100	1	.816433	.8164	.8164	0.66
	middle 100	1	.740000	.7400	.7400	0.88
	apical 100	1	.574583	.5746	.5746	0.55
	coronal 150	1	.670117	.6701	.6701	0.88
	middle 150	1	.516667	.5167	.5167	0.88
	apical 150	1	.444433	.4444	.4444	0.99
	Total	9	.697770	.4444	.9281	0.78

Table 5: CLSM values showing no significant mean difference between the groups SNI, EA, PUI and XPF when compared at coronal, middle and apical at different levels 50 μ m, 100 μ m, 150 μ m with P – value > 0.44.

There was no significant mean difference found between the groups SNI, EA, PUI and XPF when compared at coronal, middle and apical third at different levels 50 μ m, 100 μ m, 150 μ m into the dentinal tubules with P – value of 0.44 as shown in table 5 and graph 3.

Although there was no significant difference between and within the groups, at 50 μ m, 100 μ m, 150 μ m level into the dentinal

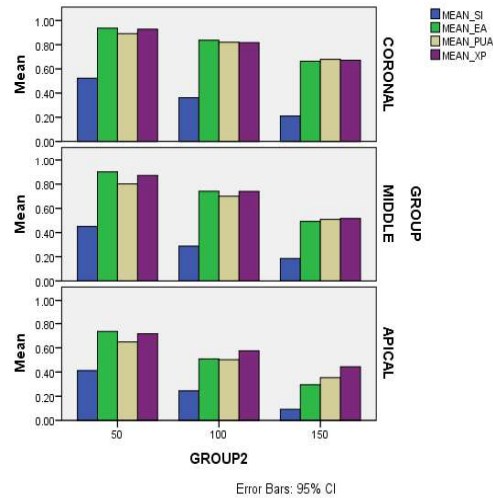
tubules, coronal third showed highest mean percentage of bacterial reduction, followed by middle and apical third in all the four groups tested, SNI (coronal third-36.47%, middle third-30.85%, apical third-24.66%), EA (coronal third-81.33%, middle third-71.33%, apical third-51%), PUI (coronal third-79.33%, middle third-66.6%, apical third-50%), and XPF (coronal third-80.33%, middle third-71.33%, apical third-58%) as shown in profile plot (Graph 2).



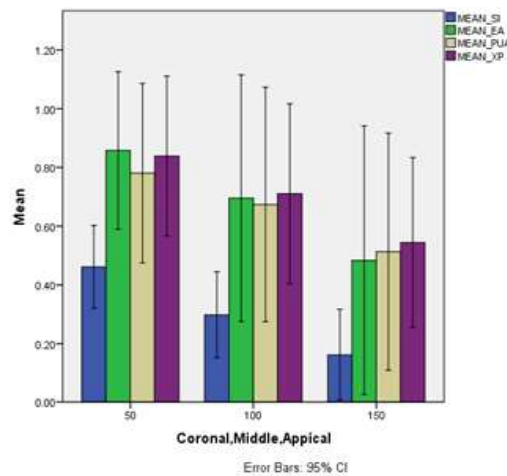
Graph 2: Profile plot showing various trends in bacterial reduction at coronal third, middle third, and apical third in four different groups. Here SI refers to SNI, EA refers to EndoActivator, PA refers to PUI, and XP refers to XPF.

As displayed in graph 3, in intergroup comparison at coronal third and middle third at 50 μm , 100 μm , 150 μm level into the dentinal tubules, SNI showed least percentage of bacterial reduction when compared to EA, PUI, and XPF with no difference among them. In apical third at 50 μm and 100 μm , SNI showed least

percentage of bacterial reduction than EA, PUI, and XPF with no difference among them. At 150 μm XPF showed highest percentage of bacterial reduction, followed by PUI and EA with no mean difference among them and SNI showed the least bacterial reduction.



A



B

Graph 3: A. Graphs showing percentage of dead bacteria at coronal, middle, and apical thirds of the canal at different depths into the dentinal tubules by different irrigant agitation techniques using CLSM analysis. B. Overall comparison between groups at 50 μm , 100 μm , 150 μm level into the dentinal tubules.

DISCUSSION

It has been well established that endodontic disease is a biofilm-mediated infection. Endodontic pathogens often form a polymicrobial biofilm where bacterial population are enclosed in a three-dimensional polysaccharide matrix, the result of which is a highly resistant and adherent infectious community. ⁽⁹⁰⁾ The organized internal compartmentalization in a biofilm allows bacteria of different growth requirements to survive in their own microenvironments. ⁽⁹¹⁾ This organization offers several advantages to bacteria, such as genetic material exchange, bacterial communication, diverse growth range, metabolic diversity, and protection from the external environment. ⁽⁹¹⁾ Therefore, the elimination of bacterial biofilms are always a challenging element to achieve successful outcome in a non-surgical endodontic treatment. Although multispecies biofilm best simulates the clinical conditions of an infected root canal system during apical periodontitis, the development of in-vitro multispecies biofilm models can be challenging. Over the past years, biofilm research in endodontics has used mainly single-species models. ^{(92), (17)} Single species biofilm of *E. faecalis* was chosen in this study because it is one of the most persistent microbial species with a prevalence up to 90% in persistent endodontic infection. ^{(8),(93)}

Extracted human natural teeth were selected in this study as human dentin is a natural substrate for biofilm growth and closely simulates the clinical situation. ⁽⁹⁴⁾ The initial step in biofilm formation is the bacterial attachment on a surface and irreversible adhesion to the substratum. Adhesion of bacteria to dentin occur more readily due to the proteinaceous portion of the dentin matrix and it seems that the use of a substrate material other than dentin does not compound the effects it has on various chemicals and disinfection techniques. ⁽³⁰⁾

This study used mandibular premolars with a single root canal. The samples were standardized in order to reduce the bias of the study. Working length was set at 18 mm, straight canal with curvature less than 5°, ratio of bucco-lingual diameter: mesio-distal diameter less than 2.5:1, and canal was instrumented with MTwo NiTi rotary files till #20/.06 to standardize apical diameter. *E. faecalis* was inoculated into the root canal till apical third as per protocol followed by Adham A. Azim et al ⁽²⁾ and Brito et al., ^{(32),(95)} *E.faecalis* was incubated for a period of 28 days in this study based on Markus Haapasalo et al ⁽³⁷⁾ and Sonja Stojicic et al ⁽⁵⁹⁾ which showed robust and consistent growth of multispecies biofilm in the canal after the 4-week incubation period ⁽³⁷⁾. Most biofilm causing endodontic and periodontal infections are likely to be several weeks, months, or even years old at the time of treatment. Therefore, when evaluating the antibacterial efficacy of

endodontic irrigants, it's important to use mature biofilms ⁽⁵⁹⁾. In this study scanning electron microscopic examination was performed to assess biofilm structure, which showed thick, matured biofilm after 28 days of incubation as shown in figure 22.

Conventionally, a chemo-mechanical approach is used to disinfect the root canal system. This method relies heavily on mechanical instrumentation to debride the pulpal tissues and biofilm from the main canal space followed by antimicrobial irrigants to disinfect bacteria and dissolve tissues. ^{(96),(97)} Sodium hypochlorite (NaOCl) is the most effective root canal irrigation solution and a powerful disinfectant that has favorable characteristics such as tissue solubility, proteolytic and bactericidal effects on microorganisms and endodontic biofilms.⁽⁹⁸⁾ the most common concentrations of NaOCl used in endodontic treatments are in the range of 0.5–5.25%. Mohammad Forough Reyhani et al evaluated the effectiveness of 2.5% and 5.25% NaOCl solution in reducing bacterial growth in *Enterococcus faecalis* biofilms in root canals and both concentrations tested showed complete inhibition of the growth of *E. faecalis*. ⁽⁶²⁾ Higher concentrations of NaOCl have shown better antibiofilm properties, but have proven to have deleterious effect on dentin matrix. NaOCl is characterized by its proteolytic action on tissue, which negatively affects dentin and causes depletion of the organic components formed mainly by type I collagen and proteoglycans. This side effect is highly undesirable

and irreversible due to the alteration of dentin structure, which causes a reduction in moisture content, consequently, leading to tooth fragility. ^{(99), (100)} Hence in the present study 3% NaOCl was used.

Aqueous solution of 17% Ethylenediaminetetraacetic acid (EDTA) is a chelating agent which is recommended as an adjuvant in root canal therapy. It is effective in removing the inorganic portion of the smear layer. ⁽⁹⁷⁾ However, EDTA has little or no antimicrobial activity according to Soares et al. ^{(41), (38)} Alternating the use of NaOCl and EDTA during root canal treatment is shown to be a more promising approach to remove the organic and inorganic debris, in addition to disrupting microbial biofilms.

Instrumentation and irrigation per se do not suffice to predictably render root canals free of cultivable bacteria. About 40%–60% of the canals still contained cultivable bacteria after chemomechanical preparation with different concentrations of NaOCl. ^{(101), (102)} This is because endodontic instruments have a design and rigidity that allow them to act only in the main canal, and NaOCl remains in the canal is rapidly neutralized by organic matter, which might be insufficient to reach other areas of the root canal system, including irregularities, lateral canals, isthmuses, apical deltas, and dentinal tubules. ⁽¹⁰³⁾ Additional irrigation agitation techniques need to be applied for efficient bacterial

elimination of biofilm from the un-instrumented areas of the canal.

(92)

Conventional irrigation with syringe and needle has been advocated as an efficient method of irrigant delivery. ⁽¹⁰⁴⁾ 30 gauge side vented needle used in this study ensured safe delivery of the irrigant to the apical third of root canal without the risk of periapical extrusion, while the dynamic up and down movement of the needle improved the hydrodynamic activation of the irrigant. ⁽¹⁰⁵⁾ Sonic and ultrasonic agitation methods has shown to be effective in achieving canal cleanliness but their results in bacterial reduction is still perplexing. ^{(74),(84),(106)} Therefore, PUI and EA were included in this study.

XP Endo Finisher (FKG Dentaire, Switzerland) is recently introduced to be used as a finishing file. There are limited studies on bacterial biofilm removal with this file. ^{(37), (2), (94), (70)} In the present study irrigant volume, time and cycle of irrigant agitation and refreshment was based on Vander Sluis et al., study. ⁽¹⁰⁶⁾ Irrigant solution was agitated in intermittent cycles rather than continuous agitation as Pingping Bao et al., study showed that intermittent agitation with irrigant solution refreshment was better than single continuous cycle of irrigant delivery and agitation. ⁽³⁷⁾

Colony forming unit (CFU) method was used in this study as it will provide information on the amount of cultivable bacteria within the biofilm structure in the main canal space.⁽¹⁰⁷⁾ Sample collection for CFU was done based on previous studies.^{(38), (89)} This study also used confocal laser scanning microscope (CLSM) to visualize the spatial distribution and calculate the ratio of live/dead bacteria at various depths into the dentinal tubules at coronal, middle, and apical segments of the root canal. The data reflect the levels of efficiency of the different irrigation protocols tested in killing the bacteria that colonized deep into the dentinal tubules.⁽²⁹⁾

This study was aimed at quantitatively and qualitatively evaluating the effectiveness of three different irrigant agitation devices in eradicating endodontic biofilm from root canals using CFU as well as in the dentinal tubules using CLSM in extracted single rooted human mandibular premolars. The null hypothesis in this study stating that there would be no statistically significant difference in eradicating *E. faecalis* biofilm from the root canals and dentinal tubules between XP-Endofinisher, passive ultrasonic irrigation and EndoActivator was accepted.

All the experimental groups showed significant reduction in CFU post instrumentation and irrigant agitation (S2) as compared to pre-instrumentation (S1) with P- value of SNI-0.05, EA-0.006, PUI-0.04, XPF-0.03 (table1). However, there was no statistically

significant difference in bacterial reduction between the four tested groups, SNI (SNI-56.40%) showed the least mean percentage reduction in CFU post intervention, followed by PUI (PUI-96.99%), and EA (EA-98.07%). The XPF (XPF - 99.94%) showed the highest percentage mean reduction in the bacteria. Previous studies have also shown comparable efficacy in *E.faecalis* eradication between EA and SNI ^{(95), (2), (108)} and PUI and SNI. ^{(69), (75), (76)} Some authors have shown better reduction in bio burden by PUI as compared to SNI. ^{(109), (77), (110), (111)} But none of them presented a similar testing condition to this study. ^{(109), (77), (111)}

According to the results of the systematic review and meta-analysis of clinical trials by Rafaela Nogueira Moreira et al., there was no statistically significant difference in the presence of bacteria between the passive ultrasonic irrigation and conventional irrigation, although a reduced quantity of bacteria has been found with the use of passive ultrasonic irrigation. ⁽¹¹²⁾

Another systematic review by Petruta E Caputa et al., concluded that Intermittent ultrasonic irrigant agitation did not improve the healing rate of apical periodontitis compared with conventional syringe irrigation after primary root canal treatment of teeth with a single root canal and also conceded that it remains unclear whether ultrasonic agitation can reduce the microbial load further than syringe irrigation in vitro. ⁽¹¹³⁾ Only a limited number

of studies are available in the literature comparing the biofilm removal efficacy of XPF with other irrigant agitation techniques. Pingping Bao et al., have demonstrated the superiority of XPF to PUI and SNI in removal of a 4-week old *E faecalis* biofilm using SEM analysis. ⁽³⁷⁾ However, SEM does not provide a reliable method for quantification of viable or cultivable bacteria within the root canal system. ⁽²⁹⁾ Alves et al., have shown that XPF resulted in significant reduction in the bacterial counts after chemomechanical preparation when compared with PUI. ⁽¹¹⁴⁾ Azim et al., have shown the XPF produced greatest bacterial reduction compared to EA, PIPS and SNI in the main canal. ⁽²⁾ The results show that the conventional SNI demonstrated nearly the same effectiveness in eliminating the bacterial biofilm from the root canal space when compared with other three systems which followed different agitation techniques. The reason for the insignificant difference between the groups might probably be attributed to the effect of irrigating solution itself and the irrigation regimen used during instrumentation of the root canal systems, which in turn could have been a major factor in reducing the bacterial count within the main canal. Similar effect has been described by P. Neelakantan et al. and Bhuva et al. showing complete destruction of biofilm irrespective of the irrigant agitation method when NaOCl was used alone or in combination with a chelator. ^{(69), (75)}

Although there was no significant difference between the experimental groups in their ability to reduce the bacterial load within the root canal, Endoactivator group showed the highest incidence of negative cultures (70%) followed by XPF (30%) and PUI (10%). However, there were no negative cultures in the SNI group. The results in the (table 3) gives the data regarding the incidence of negative cultures. Endoactivator group showed highest incidence of negative cultures compared to other groups, the reason might be due to hydrodynamic phenomenon that occurs when a vibrating tip generates fluid activation and intracanal waves. Random waves fracture, resulting in the formation of bubbles that oscillate within any given reagent. These bubbles expand, become unstable due to heat and pressure, then collapse and implode resulting in dislodgement of potential biofilms. ⁽¹¹⁵⁾ XPF Endofinisher showed 2nd highest negative cultures (30%) which might be due to the design and the helical movement of the instrument may have allowed it to reach previously untouched areas and displace bacterial biofilms.

In this study, during CLSM analysis dead bacteria were observed in samples of all irrigation groups at different depths in dentinal tubules and segments of the root that were examined. The results are in agreement with Zou et al ⁽¹¹⁶⁾ that NaOCl can manage to penetrate deeply into the dentinal tubules even without agitation. There was no statistically significant difference in percentage

bacterial reduction within and between the groups, at coronal, middle and apical third at 50 μm , 100 μm , 150 μm level into the dentinal tubules. However, the percentage of dead bacteria decreased at deeper levels of tubules; coronal third showed highest mean percentage of bacterial reduction, followed by middle and apical third in all the four groups.

This might be a result of better removal of the smear layer in coronal thirds than in apical thirds of root canals ⁽¹¹⁷⁾ and also due the morphological variations of dentinal tubules in the coronal third with a larger diameter than those in the apical area. ⁽¹¹⁷⁾

Despite the lack of statistically significant difference in the intergroup comparison, SNI showed least mean percentage of bacterial reduction at all levels and at all depths. The coronal and the middle third of all the other irrigant agitation groups showed comparable mean reduction in bacteria. In the apical third at 150 μm XPF showed highest mean percentage reduction of bacteria. The mechanical effects of the file during operation together with its irrigation agitation facilitated the removal of bacteria on the canal wall and killing of the bacteria at a depth of 150 μm . This results are in accordance with Mohammad I. Al-Obaida et al., ⁽⁹²⁾ proved that XPF improves disinfection of the root canal at all levels, especially at the apical part. On the other hand, EA had an improved performance at coronal, middle third and, apical third which may be

attributed to the maximum oscillation of the amplitude (antinode) formed at the activator tip. ⁽¹³⁾ This results is in accordance with the previous study by Jamileh Ghoddusi et al., ⁽¹¹⁸⁾ showed that the number of viable bacteria was significantly lower in the canals irrigated by Endo Activator as it can provide deeper penetration of irrigant to all areas of the canal space.

On the other hand lack of significant difference between the groups ⁽⁷⁵⁾, ⁽¹¹⁹⁾ may be due to the robust instrumentation and irrigation protocol followed in the current study when compared to other studies where teeth were instrumented to master apical file before inoculation with bacteria and irrigant agitation was performed directly after incubation without instrumentation which does not simulate clinical conditions. ⁽²⁾, ⁽¹⁰⁸⁾, ⁽⁸⁴⁾

Limitations:

1. It may be speculated that estimated sample size was not sufficient to demonstrate a statistically significant difference in the results between the groups.
2. Single species biofilm was used rather than a polymicrobial biofilm.
3. Use of single, straight root canal.
4. In the present study laboratory strain of *E. faecalis* was used, such laboratory reference strains often show important pathophysiological characteristics compared to their clinical counterparts. This is because these strains might have been sub cultured for decades since their first isolation.

SUMMARY

This study was conducted to quantitatively and qualitatively evaluate the effectiveness of three different irrigant agitation devices in eradicating *E. faecalis* biofilm from root canals and from the dentinal tubules of extracted single rooted human mandibular premolars. 64 extracted human mandibular premolars were taken and accessed, working length was determined and set as 18 mm and crowns were sectioned to standardize the root length as 19 mm. Teeth were instrumented with MTwo rotary files till #20/.06 autoclaved and three teeth were taken randomly to assess the smear layer removal by scanning electron microscope (SEM) and specimen sterility was confirmed by measuring Colony forming units (CFU). After smear layer removal and sterility assessment, *E. faecalis* (1.5×10^8 cells/ml) was inoculated into canal using sterile 1ml syringes and incubated at 37°C for 28 days at 100% relative humidity in brain heart infusion (BHI) broth to allow colonization of the bacteria on the canal wall and into the dentinal tubules. Aliquots of culture medium was replaced with fresh medium every 3 days. After 28 days of incubation three teeth were taken randomly to confirm biofilm formation. A diamond disc was used to cut a groove along the tooth's long axis stopping short of the canal lumen. A chisel was then used to split the tooth open into two pieces and then dried in ascending ethanol concentrations and sputter-coated with gold under vacuum. 56 teeth were taken and canal was

instrumented using MTwo till #35/.04, rinsed with 2 ml of 3% NaOCl with the flow rate of 2ml/min before each file and after the last file, a total of 8 ml of 3% NaOCl per canal. The teeth were then randomly divided into four groups (n = 10 teeth per group): Group 1: Conventional syringe needle irrigation (SNI), Group 2: EndoActivator (EA), Group 3: Passive ultrasonic irrigation (PUI) using Irrisafe tip, Group 4: XP Endofinisher (XPF). In each group, the canal was rinsed with 2 ml 17% EDTA for 1 minute using a 30-gauge side-vented needle, followed by flushing out the EDTA with 5ml of distilled water and then rinsing with 6 ml of 3% NaOCl for three cycles of 40 seconds with a total irrigation time of 2 minutes. Each cycle was subdivided into 20-seconds of irrigant agitation (using agitation devices according to respective groups) and 20-second refreshment. To measure the CFU samples were taken with paper points before instrumentation (S1) and after instrumentation and agitation (S2). For measuring live versus dead bacteria in the dentinal tubules (n=18) teeth were taken, divided into 4 experimental groups (n=4), instrumented and agitated as mentioned above. Two teeth were taken as controls, one tooth was autoclaved and used as a negative control. The other tooth did not receive any sort of irrigant agitation and was used as a positive control. Teeth were split open and stained with Fluorescein Diacetate (FDA) and propidium iodide (PI) before being examined under confocal laser scanning microscope (CLSM). Coronal, middle, and apical thirds of the canal dentin were scanned at 50 μ m, 100 μ m, and 150 μ m into

the dentinal tubules to determine the ratio of dead/total bacteria. Results of this study showed there was statistically significant difference in CFU between pre-instrumentation and post instrumentation with P value <0.05 . There was no statistically significant difference in percentage of bacterial reduction between the groups in the main canal space. But EA showed high incidence negative cultures (70%) followed by XPF (30%), PUI (10%), and SNI (0%). CLSM analysis revealed there was no statistically significant difference within the group and between groups at coronal, middle and apical third at all depths into dentinal tubules. But SNI showed least percentage of bacterial reduction when compared to others (EA, PUI, XPF) at all thirds of root as well as at all depths. XPF showed highest mean reduction percentage of bacteria at 150 μm in apical third. All the irrigant agitation techniques namely EndoActivator (EA), Passive Ultrasonic Irrigation (PUI), XP Endofinisher (XPF) and SNI were effective in significantly reducing the bacterial load in the main canal. Endoactivator irrigant agitation technique was able to produce more negative cultures from the main canal space compared to SNI, PUI, and XPF. None of the irrigation techniques studied demonstrated superiority over the others in their biofilm eradication ability from the main canal space as well as, at various depths within the dentinal tubules in the coronal, middle, and apical thirds, with least effect in the apical third.

CONCLUSION

Within in the limitations of the present study, it may be concluded that

1. All the irrigant agitation techniques namely EndoActivator (EA), Passive Ultrasonic Irrigation (PUI), XP Endofinisher (XPF) and SNI were effective in significantly reducing the bacterial load in the main canal.
2. Endoactivator irrigant agitation technique was able to produce more negative cultures from the main canal space compared to SNI, PUI, and XPF.
3. None of the irrigation techniques studied demonstrated superiority over the others in their biofilm eradication ability
 - From the main canal space as well as,
 - At various depths within the dentinal tubules in the coronal, middle, and apical thirds, with least effect in the apical third.
4. Dead bacteria were present at all levels and depths within the dentinal tubules irrespective of the irrigant agitation technique used.

All the root canal disinfection methods used in this study were equally effective in the elimination of *E. faecalis* biofilm from root canal, and we suggest that the irrigation regimen itself may be an important factor contributing to success.

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