

# Comparative Efficacy of Sonic, Ultrasonic, and Diode Laser-Activated Irrigation with Combination of NaOCl/EDTA/CHX Solutions Against *Fusobacterium nucleatum* in Dentinal Tubules: A Confocal Microscopy Study

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**Purpose:** This research evaluated the effectiveness of sonic, ultrasonic, and diode laser irrigation techniques with a final irrigation protocol of 3% NaOCl, 17% EDTA, and 2% CHX in eliminating *Fusobacterium nucleatum* (*F. nucleatum*) from dentinal tubules.

**Materials and Methods:** The research method utilized 38 human lower premolar teeth inoculated with *F. nucleatum*, which were divided into four groups: conventional needle irrigation (positive control), sonic irrigation (EDDY<sup>®</sup> tips), ultrasonic irrigation (IrriSafe<sup>™</sup>), and diode laser (SOLASE, 976 nm). Bacterial viability was assessed using confocal laser scanning microscopy with the LIVE/DEAD<sup>™</sup> BacLight<sup>™</sup> Bacterial Viability Kits. Data were analyzed using the Kruskal–Wallis and post-hoc Mann–Whitney tests.

**Results:** The results demonstrated that the diode laser irrigation technique achieved the highest effectiveness, with an average penetration depth of 1045.55 (740.00–1420.00)  $\mu\text{m}$ , significantly outperforming sonic (751.11 [480.00–1170.00]  $\mu\text{m}$ ;  $p$ -value = 0.024) and ultrasonic (617.77 [450.00–1090.00]  $\mu\text{m}$ ;  $p$ -value = 0.002) irrigation techniques. The control group exhibited the lowest effectiveness (243.33 [150.00–330.00]  $\mu\text{m}$ ).

**Conclusion:** Diode laser irrigation with the final irrigation protocol provided the highest efficacy against *F. nucleatum* in dentinal tubules, highlighting its superiority over other irrigation techniques for disinfection in endodontic treatment.

**Keywords:** *Fusobacterium nucleatum*, endodontic irrigation, diode laser, bacterial viability, confocal laser scanning microscopy

## Introduction

Endodontic treatment aims to eliminate microbial biofilm from the root canal system and create an environment within the root canal system that allows for healing and maintenance of the periapical tissues.<sup>1,2</sup> Failure of endodontic treatment is often due to the inability to disinfect the entire anatomy of the root canal. The primary factor contributing to the failure is the presence of persistent microorganisms, such as *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Propionibacterium* spp., which can withstand conventional disinfection methods.<sup>3–5</sup> Bacterial colonization of dentinal tubules has been extensively studied due to its role in the persistent endodontic infections, which is a major contributing factor to treatment failure in numerous cases.<sup>6</sup> Disinfection failure in endodontic procedures can be avoided by using different dynamic irrigation activation devices, such as sonic, ultrasonic, and diode laser activation devices, and the selection of appropriate irrigants.<sup>7,8</sup>

Sonic irrigation systems effectively remove biofilm primarily through the mechanism of acoustic streaming, a microfluidic flow induced by the absorption of high-amplitude oscillations but generally lack the requisite velocity and amplitude to generate cavitation.<sup>9</sup> This bubble-forming and imploding phenomenon is a key mechanism of ultrasonic activation and cannot be reliably achieved with sonic devices.<sup>10,11</sup> In contrast, diode laser irradiation has been established as a potent adjunct for endodontic disinfection. Diode laser operates at wavelengths such as 810, 940, and 980 nm and exhibits high transmission through water, enabling them to eliminate the smear layer, alter dentin morphology, and penetrate deeply into dentinal tubules. Furthermore, clinical evidence suggests that diode laser irradiation can reduce the incidence of post-operative pain following endodontic treatment.<sup>12–14</sup>

American Association of Endodontists recommends the use of irrigation materials such as sodium hypochlorite (NaOCl), chlorhexidine (CHX), and ethylenediaminetetraacetic acid (EDTA).<sup>15</sup> Sodium hypochlorite is the most commonly used irrigant due to its antimicrobial, antibiofilm, and tissue-dissolving properties. Chlorhexidine has a broad-spectrum antibacterial activity, and exhibits substantivity through its interaction with calcium ions in the dentin, resulting in a sustained release that can provide an antibacterial effect for up to 12 weeks. Meanwhile, EDTA binds inorganic materials in the root canal, which are produced by mechanical cleaning and shaping procedures in endodontic treatment. Final endodontic irrigation protocol has been recommended to use at least a combination of 3–6% NaOCl and 17% EDTA with the addition of 2% CHX in cases with extensive periapical lesions or persistent endodontic infections, combining all the benefits of these irrigants.<sup>16–18</sup>

*Fusobacterium nucleatum* (*F. nucleatum*) is prevalent in all phases of endodontic infections, including primary, secondary, and persistent endodontic infections.<sup>3,19</sup> This Gram-negative obligate anaerobic bacterium is also present in asymptomatic and symptomatic cases.<sup>6</sup> *Fusobacterium nucleatum* is known as a crucial bridging organism between primary and secondary bacterial colonies in biofilms, playing a vital role in biofilm formation.<sup>3,20</sup> Thus, *F. nucleatum* is suitable for in vitro infection studies in dentinal tubules.<sup>6,21,22</sup>

Confocal laser scanning microscopy (CLSM) is a modern imaging technology that allows researchers to view the three-dimensional structure of samples in great detail. Widely used in dentistry research, CLSM provides high-resolution images without damaging the sample, enabling in situ and real-time analysis, as well as quantitative and qualitative analysis across various layers of the sample.<sup>23–25</sup> Recently, CLSM has also been validated to assess the effectiveness of irrigants and irrigation techniques on root canal microorganisms in dentinal tubules.<sup>6</sup>

Despite the advancement of dentistry research, there are currently no studies comparing the effectiveness of various activation methods using combination of 3–6% NaOCl, 17% EDTA, and 2% CHX. Therefore, this research aimed to examine the effectiveness of sonic, ultrasonic, and diode laser irrigation techniques with a final irrigation protocol of 3% NaOCl, 17% EDTA, and 2% CHX against *F. nucleatum* in dentinal tubules, analyzed using CLSM.

## Materials and Methods

Ethical clearance was granted by the Research Ethics Committee of Universitas Padjajaran Bandung, Indonesia (No. 511/UN6.KEP/EC/2025). This study was conducted in full accordance with the ethical principles outlined in the Declaration of Helsinki. This in vitro study utilized 36 human mandibular premolar teeth, and two extra teeth were used to confirm the contamination of *F. nucleatum* into dentinal tubules. The sample size was determined using power analysis with a significance level of 0.05, power effect of 80%, and effect size of 0.6, which are generally employed in experimental research and to avoid false negatives.<sup>26</sup> The dental specimens were selected based on the following criteria: (a) Inclusion criteria, which were human mandibular premolar teeth with either a singular root, straight and mature root, or single canal, and (b) Exclusion criteria, which were human mandibular premolar teeth with either caries, calcification, or root curvature exceeding 15°.

Tooth preparation was performed according to Barros et al with minor modifications.<sup>6</sup> Briefly, all teeth were decoronated, and the apical 3 mm of each root was resected with a cutting machine (Allied Inc., TechCut 4™, Rancho Dominguez, CA, USA) at 500 rpm under constant irrigation of sterile saline solution until the root length was standardized to approximately 12 mm. The working length (WL) was determined 1 mm shorter using K-file size #10 with rubber stop, producing a working length of 11 mm. Root canal instrumentation was performed using Protaper Gold® (Dentsply Maillefer, Baillagues, Vaud, Switzerland) up to F4 (size 40, taper 06) while irrigated with 2 mL of 3% NaOCl

for 1 minute using a 30-gauge side-vented needle at 2 mm from the WL of each change of the instrument. Following instrumentation, samples were subjected to an ultrasonic bath containing a subsequent wash of 3% NaOCl, distilled water, 17% EDTA, and distilled water for 5 minutes each. The external surface of the tooth root was coated with two layers of nail polish (Revlon Super Lustrous™, Oxford, NC) while the apical region was covered with 3M™ Filtek™ Supreme Flowable Restorative in shade A2 (3M ESPE, St. Paul, MN, USA). Finally, the samples were autoclaved at 121°C and 15 MPa for 20 minutes and stored in sterile distilled water at 4°C in a biomedical refrigerator (Arctiko, PR 300®, Esbjerg, Jutland, Denmark).

The procedures used for dentin intratubular infection contamination were done as previously reported.<sup>6</sup> Briefly, strain *F. nucleatum* American Type Culture Collection (ATCC) 25586 was reactivated in trypticase soy broth (TSB) and incubated in an anaerobic chamber for 48 hours. Dental specimens were stored in pyrogenic microtubes (Eppendorf, Hamburg, Germany) with 1 mL of TSB and placed in an ultrasonic bath (Vevor Ltd, Vevor, Shanghai, China) for 15 minutes to allow maximum penetration of the broth into the dentinal tubules. Contamination was performed using a bacterial suspension of  $3 \times 10^8$  CFU/mL for 7 days with centrifugation on days two, three, five, and six. On the 7<sup>th</sup> day, the specimen was removed from the micro-pyrogenic tube and rinsed with 1 mL of phosphate-buffered saline (PBS) to remove nonadherent bacteria from the root canal walls. To ensure successful contamination of *F. nucleatum* into the dentinal tubules, observations using a scanning electron microscope (SEM) (Hitachi, SU3500, Tokyo, Japan) and confocal laser scanning microscopy (CLSM) (Fluoview FV3000, Evident Corporation, Tokyo, Japan) were conducted. Samples were initially cut longitudinally, dehydrated, and gold-stained as described in earlier report for SEM analysis.<sup>27</sup>

The dental specimens were randomly assigned into four groups: conventional needle (positive control), sonic (EDDY® tips), ultrasonic (IrriSafe™), and diode laser (SOLASE, 976 nm) groups; each group consisted of 9 specimens. The irrigation solutions used for each group were the same, as follows: 3% NaOCl, 17% EDTA, and 2% CHX, which were applied subsequently. In the control group, the root canal was irrigated with 3 mL of 3% NaOCl (1 mL/30 seconds) passively using a 30-gauge side-vented needle, followed by 2 mL of sterile distilled water. Next, the root canal was passively filled with 3 mL of 17% EDTA (1 mL/30 seconds) solution and then irrigated with 2 mL of sterile distilled water. The root canal was then irrigated with 3 mL of 2% CHX (1 mL/30 seconds) solution and rinsed with sterile distilled water.<sup>17</sup>

In the sonic group, the root canal was passively filled with 3% NaOCl using a 30-gauge side-vented needle placed at the orifice. Irrigation of the root canal was activated using a sonic tip (VDW GmbH, EDDY® tips, Munich, Bavaria, Germany) attached to an airscaler (Nakanishi Inc. NSK S970, Kanuma, Tochigi, Japan). The sonic tips were placed 2 mm from the WL of the root canal with vertical movements. The root canal was activated for 30 seconds with 1 mL of 3% NaOCl (1 mL/30 seconds), followed by a 30-second soaking interval. This cycle was repeated twice, then irrigated with 2 mL of sterile distilled water. The 17% EDTA and 2% CHX irrigation solutions were applied sequentially using the same procedures as the first irrigation solution, with each step ending with 2 mL of sterile distilled water irrigation.

In the ultrasonic group, the root canal was passively filled with 3% NaOCl using a 30-gauge side-vented needle placed at the orifice. Root canal irrigation was activated using an ultrasonic file K25/21mm (Acteon, IrriSafe™, Mérignac, Gironde, France) mounted on an ultrasonic machine (Acteon, NEWTRON® p5XS, Mérignac, Gironde, France) and positioned 2 mm from the WL with a power setting of 7. The root canal was activated for 30 seconds with 1 mL of 3% NaOCl (1 mL/30 seconds), followed by a 30-second soaking interval. This cycle was repeated twice, then irrigated with 2 mL of sterile distilled water. A solution of 17% EDTA and 2% CHX was applied sequentially using the same procedures as the first irrigation solution, with each step ending with 2 mL of sterile distilled water irrigation.

Lastly, in the diode laser group, the root canal was passively filled with 3% NaOCl using a 30-gauge side-vented needle placed at the orifice. Root canal irrigation was activated using a diode laser (Lazon Medical Laser Co., Ltd, SOLASE, Hunnan, Liaoning, China) with a flexible optical fiber tip of 200 µm diameter, set at 1 W in pulse mode, positioned 2 mm from the WL. The active power output was measured using a power meter, and the laser tip was set at a 45° angle to ensure consistent laser application. The root canal was activated for 20 seconds with a backward helicoidal movement at a speed of 2 mm/second using 1 mL of 3% NaOCl (1 mL/20 seconds), followed by a 30-second soaking interval. This cycle was repeated twice, then irrigated with 2 mL of sterile distilled water. A solution of 17% EDTA and

2% CHX was applied sequentially using the same procedures as the first irrigation solution, with each step ending with 2 mL of sterile distilled water irrigation.

After irrigation procedure, bacterial viability was examined using CLSM.<sup>6</sup> The specimens were initially embedded in resin (Vertex-Dental, Vertex<sup>®</sup> Castapress, Soesterberg, Utrecht, the Netherlands) for structural stabilization and transverse sectioning at the apical third for standardized evaluation of bacterial viability into 500 µm-thick sections using a sterile diamond disc under constant irrigation of sterile distillate. The sections were washed with 17% EDTA for 3 min, followed by sterile distillate to eliminate the smear layer resulting from the cutting procedure. The samples were stained using the LIVE/DEAD<sup>™</sup> BacLight<sup>™</sup> Bacterial Viability Kits (Thermo Fisher Scientific Inc., Molecular Probes, Waltham, MA, USA), containing SYTO 9<sup>™</sup> Green Stain and Propidium Iodide Red Stain<sup>™</sup>, for 20 minutes according to the manufacturer's instructions. The samples were examined using an Fluoview FV3000 confocal laser scanning microscope (Evident Corporation, Tokyo, Japan), with a wavelength setting of 480/500 and 490/635 nm for SYTO 9 (green nucleic acid color) and propidium iodide (red nucleic acid color), respectively. Bacteria with intact cell membranes appeared green, while bacteria with damaged membranes appeared red. The buccal-palatal region was determined by drawing a median line, and images were taken from the region for each sample. Images were then taken at magnifications of 1.25x and 10x, with 1024 × 1024 pixels resolution. The images obtained were fragmented and converted to TIFF format. The red fluorescence depth was expressed in µm and measured using cellSens FV software (Evident Corporation, Tokyo, Japan) at 1.25x magnification. The CLSM results were quantitatively analyzed to evaluate the penetration depth of the irrigants and their antimicrobial efficacy based on the viability of *F. nucleatum*.

Statistical analysis was performed using IBM SPSS version 25 (IBM, New York, USA). Data normality was evaluated using the Shapiro–Wilk test. If the data were normally distributed, a one-way analysis of variance (ANOVA) with post-hoc Bonferroni was performed. If the data were not normally distributed, the Kruskal–Wallis test was used, followed by post-hoc Mann–Whitney test.

## Results

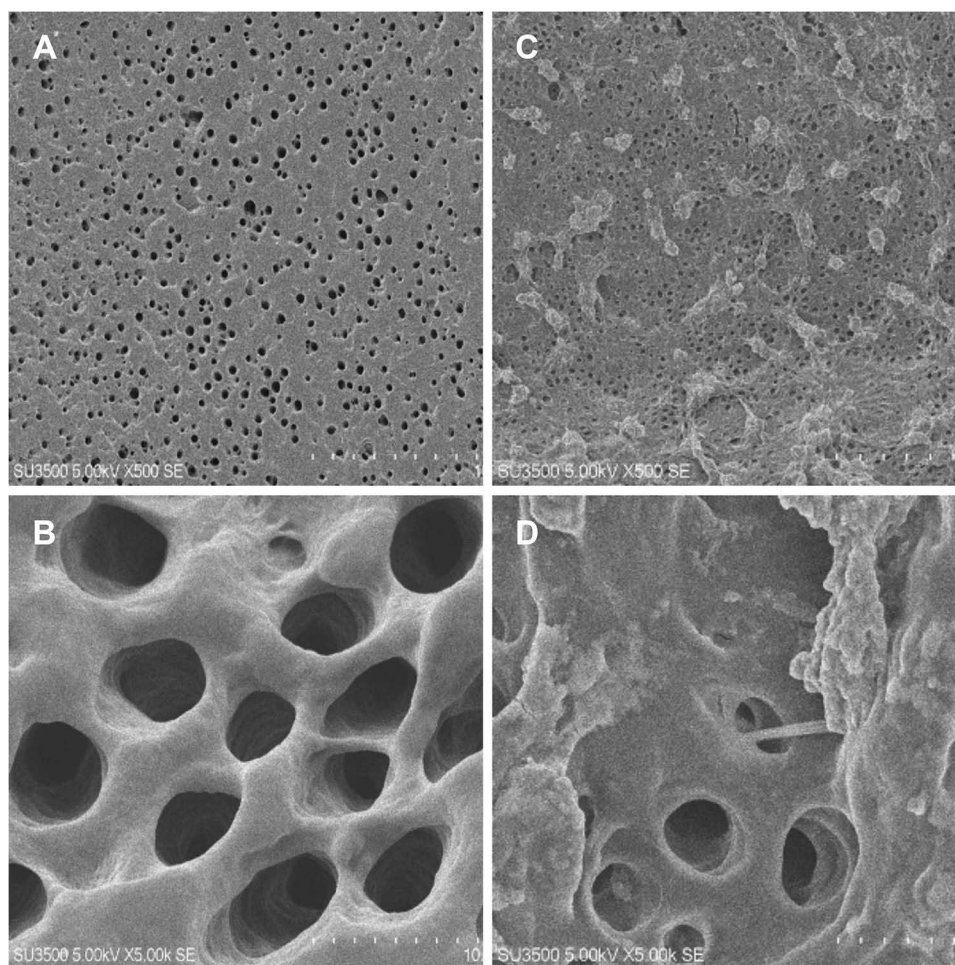
Scanning electron microscope results showed absence of microbial contamination with clean dentinal tubules before dentin intratubular contamination protocol (Figure 1A and B) and successful contamination into the dentinal tubules, as evidenced by the presence of bacteria on the dentin surface and within the dentinal tubules (Figure 1C and D). Successful of *F. nucleatum* contamination into the dentin tubules was also confirmed by CLSM results, where the penetration depth of *F. nucleatum* contamination reached over 1000 µm with intact membrane integrity, indicated by green fluorescence signals within the dentin tubules (Figure 2A). CLSM scanning did not detect red fluorescence in the dentinal tubules, indicating the absence of bacteria with damaged membrane integrity (Figure 2B).

The effectiveness of irrigation techniques against *F. nucleatum* in dentinal tubules are shown in Figure 3. After the Shapiro–Wilk test, it was found that the data were not normally distributed. Therefore, the data were presented as means with minimum and maximum values. The conventional needle irrigation technique had the lowest effectiveness value of 243.33 (150.00–330.00) µm. The ultrasonic and sonic irrigation techniques had higher effectiveness values than the conventional needle irrigation technique, with values of 617.77 (450.00–1090.00) µm and 751.11 (480.00–1170.00) µm, respectively. The diode laser irrigation technique had the highest effectiveness value compared to the other three techniques, with a value of 1045.55 (740.00–1420.00) µm (Figure 4A–D).

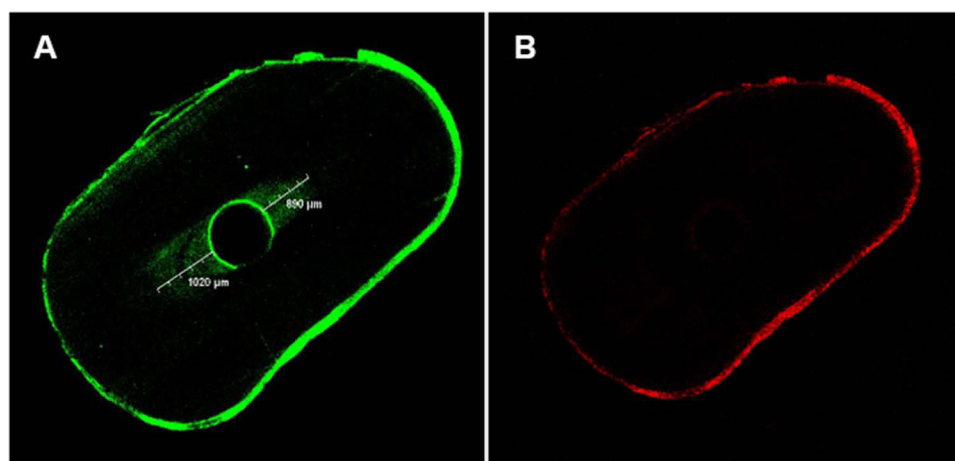
Further data analysis was performed using the Kruskal–Wallis test with post-hoc Mann–Whitney (Table 1). There were significant differences between diode laser irrigation and sonic (p-value = 0.024), ultrasonic (p-value = 0.002), and conventional needle irrigation (p-value < 0.001) techniques, with diode laser showing significantly higher efficacy. The effectiveness of sonic and ultrasonic irrigation techniques was significantly greater than that of conventional needle irrigation (p-value < 0.001). However, no significant difference was found between ultrasonic and sonic irrigation techniques (p-value = 0.269).

## Discussion

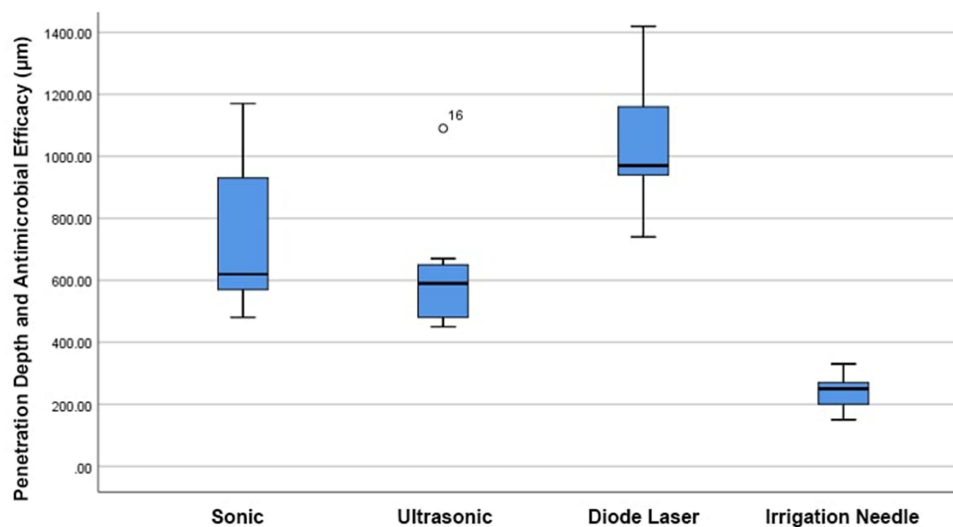
The success of root canal treatment is partly determined by clean root canals free of debris and microbial infection, which can be achieved through root canal irrigation using the appropriate irrigants. The study results show that the diode laser



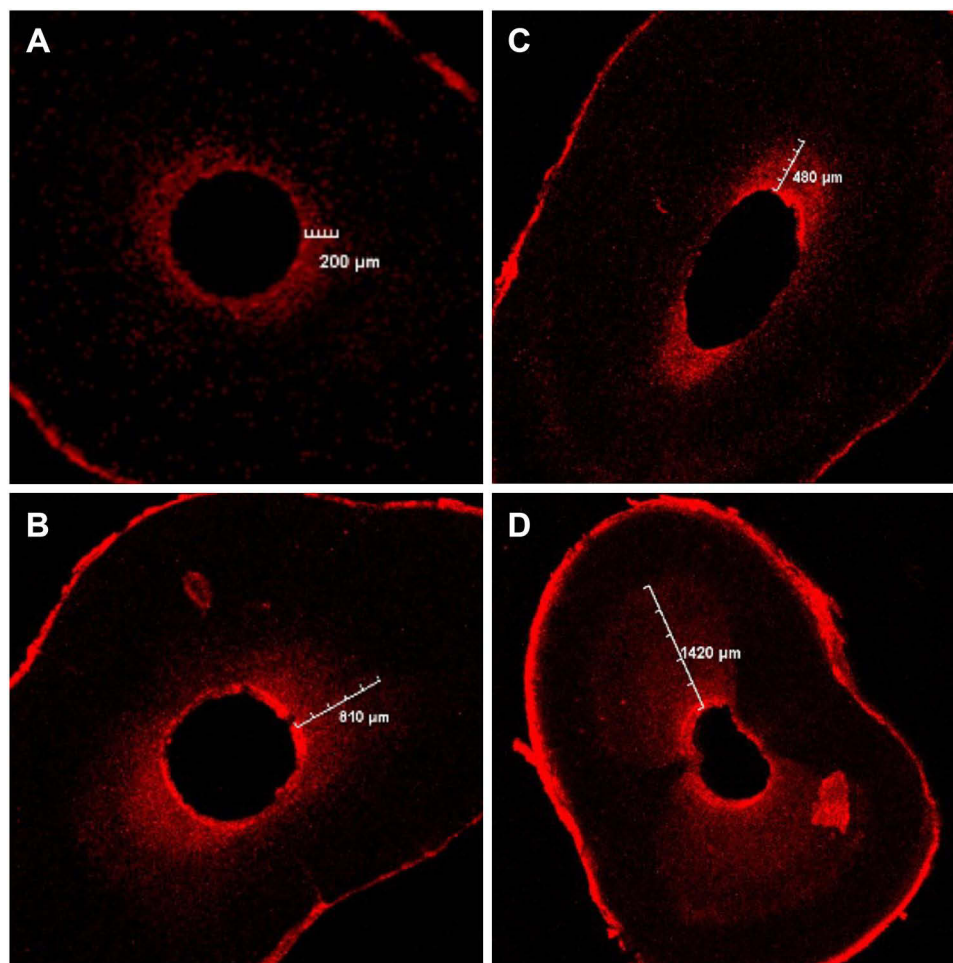
**Figure 1** Representative images of scanning electron microscope results displaying dental specimens before (A and B) and after (C and D) *F. nucleatum* contamination taken at 500x (top) and 5000x (bottom) magnifications.



**Figure 2** Representative images of confocal laser scanning microscope results after *F. nucleatum* contamination under SYTO 9 (A) and propidium iodide (B) staining at 1.25x magnification.



**Figure 3** Comparative evaluation of irrigation techniques on the elimination of *F. nucleatum* from dentinal tubules.



**Figure 4** Representative images of confocal laser scanning microscope results following irrigation needle (A), sonic (B), ultrasonic (C), and diode laser (D) irrigation techniques at 1.25x magnification.

**Table 1** Post-Hoc Mann–Whitney Test Results of the Mean Effectiveness of Four Irrigation Techniques with Final Irrigation Protocol on the Viability of *F. nucleatum* in Dentinal Tubules

No	Group 1	Group 2	$\Delta$ Mean (Group 2 – Group 1)	P-value
1	Sonic	Ultrasonic	–133.34	0.269
2	Sonic	Diode laser	294.44	0.024
3	Sonic	Needle irrigation	–507.78	<0.001
4	Ultrasonic	Diode laser	427.78	0.002
5	Ultrasonic	Needle irrigation	–374.44	<0.001
6	Diode laser	Needle irrigation	–802.22	<0.001

irrigation technique is the most effective technique compared to the other irrigation techniques in in vitro test on human mandibular premolar teeth inoculated with *F. nucleatum*.

Among the four irrigation techniques tested, the conventional needle irrigation technique exhibited the lowest effectiveness, as it could only reach an average depth of 243.33  $\mu$ m, consistent with previous studies suggesting that the irrigation penetration of this technique could not penetrate dentin tubules beyond 300  $\mu$ m, especially in the apical region of the tooth.<sup>28</sup> Additionally, this technique was reported to be insufficiently effective in reducing bacterial load and experienced a decrease in effectiveness, particularly in the root canal areas that are difficult to reach.<sup>29</sup> The limitations of this technique are influenced by root canal morphology, needle placement depth, needle diameter, and needle hole position, leading to variations in apical pressure during irrigation. The use of this irrigation technique may have a negative impact on the prognosis of root canal treatment and, in some cases, contribute to persistent apical inflammation.<sup>30–32</sup>

Similar effectiveness between sonic and ultrasonic irrigation techniques has also been reported in previous studies, in which there were no significant difference in terms of bacterial load reduction, debris removal, and organic tissue cleaning.<sup>33–35</sup> When the EDDY<sup>®</sup> sonic irrigation system, driven by an airscaler, operates at a frequency of 6000 Hz, it generates high-frequency vibrations that trigger physical effects such as cavitation and acoustic streaming, previously known to be induced only by ultrasonic irrigation technique.<sup>35,36</sup> The combination of acoustic streaming and cavitation is highly effective in thoroughly cleaning root canals and reducing the risk of further infection.<sup>34,35</sup>

Our results in this research demonstrate that the diode laser irrigation technique is the most effective technique to eliminate *F. nucleatum* in dentinal tubules compared to the other three techniques. These findings align with the in vivo study by Fahim SZ, Ghali RM, Hashem AA, and Farid MM (2024), which found that the technology significantly reduced the population of aerobic and anaerobic bacteria by up to 99.94%.<sup>37</sup> Diode laser is a type of near-infrared (NIR) laser with low absorption of hydroxyapatite crystals or water. This absorption capability allows the diode laser to penetrate deeper into the dentinal tubules and exert its antibacterial effect. The diode laser produces a photodisruptive effect on bacteria that cannot be reached when direct cell death does not occur, resulting in antibacterial activity.<sup>37,38</sup> The diode laser has been proven effective in root canal disinfection, with better penetration capabilities, enabling it to reach difficult parts of the root canal.<sup>39,40</sup>

This study utilized a low-power diode laser, specifically 1W, in pulse mode at 100 ms for 20 seconds, with a flexible optical fiber tip of 200  $\mu$ m diameter, providing flexibility in accessing complex root canals. The longer exposure time at lower power allows for more controlled absorption of laser energy, thereby preventing damage to the surrounding tissues. Additionally, the diode laser enables deeper, broader, balanced, and controlled penetration of the laser beam throughout the root canal walls in three dimensions, supported by backward helicoidal movement that optimizes the disinfection process of all dentinal tubules. Lower power has also been reported to reduce pain or discomfort in patients.<sup>41</sup>

This research further strengthen the use of diode laser irrigation technique with final irrigation protocol for root canal treatment over other irrigation techniques. Further studies are needed to explore various modes and power settings to determine the optimal settings. Although this study was well designed, it has limitations as it only used one type of bacteria. This condition does not adequately represent the clinical conditions of root canal infections, which involve polymicrobial biofilms composed of various bacterial species interacting in a complex manner.<sup>42,43</sup>

This study is limited by its inability to quantify the ratio of viable to non-viable bacteria, as our confocal microscope (Fluoview FV3000) lacks a dedicated photon counting detector, which is essential for the precise signal discrimination required for this quantitative analysis.

## Conclusion

Diode laser irrigation technique with final irrigation protocol is the most effective irrigation approach in eliminating *F. nucleatum* from dentinal tubules, surpassing conventional needle, sonic, and ultrasonic irrigation techniques. This finding supports the use of diode laser in clinical setting over other techniques. Further research is needed to determine the optimal mode and power for diode lasers. In addition, testing of polymicrobial biofilm samples in dentinal tubules is necessary to improve clinical relevance, taking into account parameters such as the penetration depth of the irrigants, antibiofilm activity, and dentin preservation. Furthermore, utilizing advanced quantitative confocal microscopy techniques, such as photon counting, to provide a precise ratio of viable to non-viable bacteria within defined regions of interest will add a great value to this study.

## Ethics and Consent

This in vitro study utilized extracted human teeth. Informed consent was obtained from all participants prior to the study. The protocol for the use of these teeth was reviewed and approved by the Research Ethics Committee of Universitas Padjadjaran (Ethical Approval Number: 511/UN6.KEP/EC/2025). All patient identifiers were removed to ensure complete anonymity.

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## Disclosure

The author(s) report no conflicts of interest in this work.

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