



# Comparison of the *Enterococcus faecalis* Colony Reduction Effect of Two Wavelengths of Diode Lasers With Three Methods of Root Canal Irrigation: An In Vitro Study

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## Abstract

**Introduction:** The basis of successful root canal therapy is the reduction of microorganisms. The aim of this in vitro study was to compare the antibacterial effect of three different irrigation methods with two laser wavelengths on *Enterococcus faecalis* biofilm.

**Methods:** Ninety-five single-canal teeth were prepared, sterilized, and divided randomly into a negative control, a positive control, and five test groups. They were inoculated with the standard strain of *E. faecalis*. The test groups were conventional irrigation (group 1), Passive ultrasonic irrigation (group 2), Gentle file finisher brush (group 3), 810 nm diode laser (group 4), and 980 nm diode laser (group 5). Microbial sampling, cultivation, and colony counting were done. Data were analyzed with the Kruskal-Wallis test and a negative binomial regression model.

**Results:** There was a significant difference in the colony count between the groups ( $P < 0.001$ ). The 810 nm diode had the highest and the conventional irrigation group had the lowest reduction in the microbial load. Passive ultrasonic, 980 nm diode laser, and Gentle file finisher brush groups were also ranked respectively from the highest to the lowest in terms of decreasing effect on the number of colonies.

**Conclusion:** The 810 nm diode laser and conventional irrigation were respectively the most and the least effective methods for reducing the number of *E. faecalis* colonies.

**Keywords:** Diode laser; Root canal therapy; *Enterococcus faecalis*.



## Introduction

As the success of root canal treatment depends on the effectiveness of the process of cleaning and shaping the canal, the primary goal of endodontic treatment is to disinfect the canal and the three-dimensional network of dentinal tubules.<sup>1,2</sup> In fact, the persistence of bacteria in this complex anatomical structure can be considered the main cause of treatment failure. The morphology of the canal makes the mechanical preparation face limitations. In addition, chemical irrigants can only be effective in the dentin layers that are directly adjacent to the canal wall, and their penetration in the dentin is limited to about 130  $\mu\text{m}$  while bacteria can penetrate up to 1000  $\mu\text{m}$  in the dentin.<sup>3</sup>

Therefore, to overcome these limitations, the use of manual or machine activation irrigation methods is a way to allow chemical solutions to penetrate deeper and remove bacteria from the inaccessible areas of the canal system.<sup>4</sup> The ultrasonic system, which has two types

including ultrasonic irrigation (UI) and passive ultrasonic irrigation (PUI), is one of these activation systems.<sup>5,6</sup> The first type (UI) is a combination of simultaneous ultrasonic instrumentation and irrigation while the second one (PUI) operates without any instrumentation.<sup>7</sup> Although some studies showed better cleaning ability of the canals prepared by the UI method than the conventional preparations,<sup>8-11</sup> others fail to show its superiority.<sup>12-14</sup> These results are probably related to the limitation of vibratory motion and the cleaning efficiency of the ultrasonic file inside the un-instrumented canal space as well as the lack of control of the amount of dentin cutting and the risk of root strip perforation.<sup>15-18</sup> Therefore, current articles support the use of ultrasonic in a passive way and as a complement to canal preparation.<sup>19</sup> Another method that has recently been introduced to improve canal preparation, eliminate remaining bacteria, and prevent persistent and secondary infections, especially in oval canals, is the Gentle File (GF) system. The main difference between these instruments

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and rotary NiTi instruments is that they do not cut into the dentine, but rather abrade/scrape the dentinal walls. The Finisher GF Brush works by opening its six SS fine strands when rotated. This may scrape the canal walls to remove tissue and microbial biofilms that are attached more effectively than syringe irrigation.<sup>20</sup>

In addition, a relatively new technology to complete the disinfection process of the root canal is to use a laser.<sup>21</sup> The major benefits of this method as an adjunctive to conventional chemical and mechanical techniques are its ability to penetrate the dentin adjacent to the canal and its antibacterial properties.<sup>22,23</sup> That means the excellent bactericidal effect of the diode laser is due to its greater penetration depth (up to 1000 microns in dentinal tubules) compared to the limited penetration power of chemical disinfectants.<sup>24</sup> In other words, its inherent characteristics, such as light scattering, local intensity enhancement, and attenuation, allow deeper penetration and reach areas that cannot be reached by conventional methods.<sup>25,26</sup> In addition, absorbed laser energy can be converted into thermal energy and cause tissue changes. The total amount of laser energy that can be absorbed depends on its wavelength and the optical properties of the target tissue, including its pigmentation and water content.<sup>21,27,28</sup>

*Enterococcus faecalis* is the most common cocci involved in resistant and recurrent infections. This is because of its ability to resist against sodium hypochlorite by forming biofilm and penetrating dentinal tubules and also against alkaline pH of calcium hydroxide because of its proton pump in its cell membrane.<sup>29</sup>

In evaluating the antibacterial effect of diode lasers, some studies showed that in comparison with other methods such as using Endovac, PUI, and conventional techniques, diode lasers can act more effectively to reduce the bacterial load.<sup>1,30,31</sup> But since few studies have compared the disinfection efficiency of different wavelengths of these types of lasers with other newer and older methods, this comparison was the aim of this study. Also, considering the contradictory nature of the studies on the disinfection effects of PUI and diode lasers, as well as the small number of studies on the GF system in this field and to choose the best and most effective method to remove the root canal bacteria, this study was done.

## Materials and Methods

This in vitro study (ethic code: IR.IAU.KHUISF.REC.1400.015) was conducted in 2021-2022 at the Endodontic Department of Dental School of Islamic Azad University, Isfahan (Khorasgan) Branch, Iran. Ninety-five human single-canal and single-root teeth, which were scheduled for extraction due to periodontal disease, prosthodontic or orthodontic purpose,<sup>32</sup> were collected. After taking two analog radiographs in the mesiodistal and buccolingual dimensions of each tooth, the teeth with

open apices, caries, cracks, calcified canals, anatomical variations or irregularities and the single roots with two canals were excluded from the study. In addition, as the teeth should have a standard root length of 15 mm<sup>1</sup> after crown cutting, longer or shorter cases were also excluded. The root surfaces of the selected teeth were debrided by a periodontal curette, immersed in 5.25% sodium hypochlorite for one hour, and kept in 0.9% sterile saline until preparation.

To cut the crown, first, each tooth was precisely marked with a caliper (TRICLE BRAND, China) to the desired length and then cut from the marked place using a safe-sided diamond disc (MICRODONT, Brazil) and a micro-motor (MARATHON, ESCORTIII, South Korea). Thereafter, a #15 hand K-file (Dentsply Maillefer, Tulsa, OK, USA) was used to determine the working length of the canal by subtracting 0.5 mm from the length of the file when it extruded just beyond the apical foramen which was verified with a dental microscope (ALLTION AM-2000, Netherlands) with three times magnification.<sup>32,33</sup> An analog radiograph was also taken to confirm the obtained length of the teeth.

Cleaning and shaping were done for each tooth by using assorted Goldent (Goldent file, China) rotary files (350 rpm and 3 N/cm torque) and Eighteeth-E connect Pro Cordless Endo Micromotor (Eighteeth, China) up to size F<sub>2</sub> (D0 = 0.25 mm). 3 ml of 2.5% sodium hypochlorite was used as the irrigant between each instrument changing by using a side-vented needle. Finally, the canals were irrigated with 3 ml of 17% EDTA solution for 1 minute, 3 ml of 2.5% sodium hypochlorite, and 3 ml of sterile saline (sodium chloride 0.9 %), respectively.<sup>1</sup> The purpose of using EDTA was to remove the smear layer for better colonization of dentine tubules with *E. faecalis*.<sup>1</sup>

To prepare samples for sterilization, after drying the canals with #25 sterile paper points, the apices of the roots were sealed by using cyanoacrylate.<sup>1</sup> Furthermore, root surfaces were covered with two layers of nail varnish.<sup>32</sup> Each tooth was transferred into a test tube containing sterile brain heart infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) and autoclave-sterilized at 121 °C with 15 Psi pressure for 30 minutes.<sup>32</sup> Five teeth were randomly selected as the negative control group and incubated in BHI broth for 24 hours. No bacterial growth in the negative control tubes indicated the absence of contamination.<sup>32</sup>

For the inoculation of *E. faecalis* bacteria in dental samples, first the frozen standard strain of *E. faecalis* (ATCC 9854) was transferred to the BHI-agar (Merck KGaA, Darmstadt, Germany) solid culture medium; after incubation at 37 °C for 24 hours with 5% CO<sub>2</sub> under anaerobic condition, 0.5 McFarland's standard concentration was prepared, which includes 1.5 × 10<sup>8</sup> CFUs/mL. Then, 100 µL of this suspension was injected into each canal by means of a sterile insulin syringe.<sup>32</sup> The

canals were then incubated under anaerobic conditions at 37 °C for 3 weeks to form *E. faecalis* biofilm in the root canal surfaces.<sup>1</sup> The culture medium of the tubes was refreshed daily.

After the incubation time finished, the samples were divided into 5 test groups and a positive control group as follows (n=15 each):

1. The conventional irrigation group using a 30-gauge side-vented needle without activation;
2. The PUI group using U-file to agitate irrigant solutions;
3. The Gentle file finisher brush group which was used for agitation and activation;
4. The 980 nm diode laser group;
5. The 810 nm diode laser group.

The positive control group did not receive any treatment to indicate the initial number of bacteria and serve as a benchmark for comparison.<sup>1</sup> The negative control group, as mentioned before, included five teeth that did not grow any microorganisms after sterilization.

In all groups, first, the remaining BHI-broth in the canals was dried with #25 sterile paper points. Then, the canals were prepared again up to size F<sub>4</sub> (D<sub>0</sub>=0.4 mm) of Goldent rotary files. In this level, the irrigant solution which was used in all study groups between each file number was 1 mL of 5.25% sodium hypochlorite. Moreover, final irrigation was done with 5 mL of 17% EDTA, 5 mL of 5.25% sodium hypochlorite, and 5 mL of sterile saline respectively in each group in the following order:

In the conventional irrigation group, a 30-gauge side-vented needle was passively placed 1 millimeter shorter than the working length between changing each file number, and the irrigation was done with 1 mL of 5.25% sodium hypochlorite. In the end, the final irrigants mentioned above were injected into the canals for 2 minutes.<sup>1</sup>

In the passive ultrasonic group, at first, 5 mL of EDTA entered the canal. After that, 2 mL of hypochlorite was injected for 30 seconds. Then, for 20 seconds, activation was done by using #15 U-file (Woodpecker, China) while the ultrasonic device (Varios 970, NSK, France) was set at a quarter of the maximum power and endodontic mode. Again, 1 mL of hypochlorite was injected into the canal with a side-vented needle for 20 seconds, and after that, activation was done for 20 seconds. Finally, the remaining 2 mL of hypochlorite was injected into the canal for 30 seconds<sup>4</sup> and the final irrigation was done with 5 mL of sterile saline.

In the Gentle file group, after preparation as in other groups, first EDTA and then sodium hypochlorite entered the canal. After that, the finisher brush (mib, South Korea) was placed one millimeter shorter than the working length and was used in up and down motion according to the manufacturer's instructions for 2 minutes to activate

sodium hypochlorite.<sup>20,34</sup> Finally, the canal was irrigated with sterile saline.

In the laser groups, final irrigants were used in the same way as the conventional irrigation group, that is, first EDTA, then sodium hypochlorite, and sterile saline thereafter. Lastly, the canal was dried with #40 sterile Paper point. The laser power was set to 1.5 W and the laser radiation was performed with the help of flexible and disposable fiber optics of 200 microns in the 980 nm diode laser (Pulsar, Iran) and 300 microns (because 200 micron fiber was not available at the research site) in the 810 nm diode laser (Doctor Smile, Italy). Thus, the fiber was 1 mm shorter than the working length and the device was used in continuous wave mode.<sup>32</sup> The teeth were subjected to four cycles of diode laser irradiation, with 5 seconds of irradiation and 20 seconds of resting period. The handpiece of the laser was held to make an angle of 10 degrees with the longitudinal axis of the tooth. The laser was used in a circular motion in the apical to the coronal direction without water or any type of cooling. In the end, some saline was injected into the canals as a transfer medium to facilitate microbial sampling.<sup>32</sup>

After applying the above methods on the teeth, microbial sampling was done from inside the canals with the help of a #4 Peso Reamer (Mani, Japan) (with ISO size equivalent to 130) to transfer the dentine shavings to micro-tubes (Eppendorf, Germany) containing 1 ml of saline for each root canal. Then, three #40 sterile paper points were also used to transfer the remaining liquid to the corresponding tubes of each root.<sup>35</sup>

Then, the tubes were transferred to the laboratory. After preparing the dilutions, the colonies were cultured on Mueller Hinton agar (Merck KGaA, Darmstadt, Germany) plates and incubated for 48 hours at 37 °C under anaerobic conditions. After that, the colonies were counted. The colony count formula is the product of the number of colonies multiplied by the inverse of the dilution factor inoculated inside the plate (Figure 1).

Finally, the values were compared to the number of initial colonies before starting the procedures and applying the methods, which was the same number of colonies in the positive control group, and the data were analyzed by using the Kruskal-Wallis non-parametric test, the negative binomial regression model, and SPSS software version 22.

## Results

Among all study groups, the lowest number of colonies in the 810 nm diode laser group ( $279.33 \pm 173.924$ ) and the highest number of colonies belonged to the positive control group ( $30066666.67 \pm 9647106647.419$ ) (Table 1 and Figure 2).

In comparing the amount of *E. faecalis* colonies based on the Kruskal-Wallis test, the number of *E. faecalis* colonies was not the same among the 6 groups and there



Figure 1. (a) Colonies grown on the environments of the positive control group, (b) Colonies grown on one of the environments of the studied groups

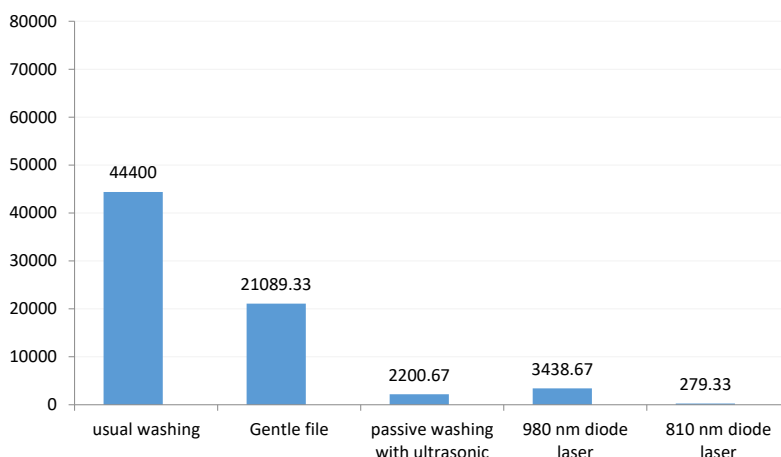


Figure 2. Comparison of the Mean Number of Colonies (CFU/mL) of *Enterococcus faecalis* in 6 Study Groups

Table 1. Comparison of the Number of Colonies (CFU/mL) of *Enterococcus faecalis* in 6 Study Groups

Group	No.	Mean	SD	Minimum	Maximum	P Value
Positive control	15	30066666.67	9647106647.419	$2 \times 10^{10}$	$5 \times 10^{10}$	<0.001
Usual washing	15	44400.00	27590.889	20000	110000	
Gentle file	15	21089.33	28992.817	240	81000	
Passive washing group with ultrasonic	15	2200.67	3168.207	100	12500	
980 nm Diode laser	15	3438.67	2736.507	350	7400	
810 nm Diode laser	15	279.33	173.924	0	680	

was a statistically significant difference between the groups (Table 1).

In a two-by-two comparison of the number of colonies of *E. faecalis* between the positive control group and other groups based on the negative binomial regression model, the average number of colonies in the positive control group was higher than in the other groups, and it was a positive decrease in the incidence rate of *E. faecalis* colonies in all 5 groups compared to the control group ( $P < 0.001$ ).

Also, in the two-by-two comparisons, when comparing the number of colonies of *E. faecalis* in the conventional irrigation group, in the GF group, in the PUI group, in the 980 nm diode laser group and in the 810 nm diode laser group with other groups, the differences in the colony occurrence rate were significant ( $P < 0.05$ ) and the 810 nm diode laser group had the highest reduction in all these

comparisons. The only exception was the difference in the incidence rate of the colony between the PUI group and the 980 nm diode laser group, which was not significant ( $P = 0.222$ ).

Thus, in general, all the test groups have shown a decrease in the number of colonies compared to the positive control group. In addition, the greatest decrease in the incidence rate was observed in the 810 nm diode laser group, which means it was more effective than the 980 nm type, and this group had the best reduction effect on *E. faecalis* bacteria (Figure 1). Furthermore, generally, the groups of PUI, 980 nm diode laser, Gentle file, and conventional irrigation were placed in the next categories of effectiveness, respectively from more to less, but in the two-by-two comparisons, the differences were not significant between the 980 nm diode laser and PUI groups.

## Discussion

The purpose of this study was to determine and compare the antibacterial effect of the conventional irrigation method with a side-vented syringe and other irrigation or activation methods including PUI, final irrigation with the finishing brush of the Gentle File system, and 810 and 980 nm diode lasers.

Since *E. faecalis* is frequently seen in connection with persistent endodontic infections, it can grow in biofilm form, and its three-week biofilm has high resistance compared to younger biofilms. This bacterium was selected as the target organism.<sup>1</sup>

The antibacterial effect of the PUI method can be attributed to the energy produced, which improves the canal disinfection ability through cavitation, acoustic streaming, and heating of sodium hypochlorite.<sup>33</sup> Furthermore, it seems that the mechanism of the effect of the GF system on any bacteria is to reduce the amount of possible residual pulp tissues and microbial shelters, as well as overcoming the insufficiency of the hydrodynamic shear stress of the syringe and needle to separate the biofilms attached to the canal wall.<sup>36,37</sup> On the other hand, the lethality of the laser is basically due to the thermal heating of the environment above the lethal value and local heating inside the bacteria.<sup>29,38</sup> However, because *E. faecalis* is very resistant to heat due to the structure of its cell wall, other characteristics of the laser in this particular species should be considered the main responsible for the anti-bacterial effect. In the explanation of this issue, it should be mentioned that the near-infrared lasers (from 810 nm to 1340 nm) which include a diode laser have negligible affinity for water and the hydroxyapatite of hard dental tissues, and therefore, they penetrate largely into dentinal tubules and are absorbed by the bacteria pigments. This allows for a bactericidal effect in deeper dentin layers.<sup>39-42</sup>

According to the results of the present study, regular irrigation with a syringe has the least effect on the removal of *E. faecalis* bacteria compared to the use of diode laser, PUI, and Gentle file finisher brush, which is consistent with the results of other studies.<sup>30,43</sup>

One of the reasons for this low effect is the insufficient penetration of the detergent in the canal system; thus, the highest flow rate is established only in the lumen and around the tip of the needle, and this issue makes it difficult for sodium hypochlorite to reach the inaccessible areas.

Also, the high surface tension of sodium hypochlorite prevents its direct contact with the dentine walls of anatomical complexities. The irrigant agent only creates a limited effect beyond the tip of the needle, and the reason for this should be found in the presence of the dead-water zone or sometimes air bubbles (apical vapor lock) in the apical part of the canal, and this prevents the apical penetration of the irrigant solution.<sup>30</sup>

In comparison with other methods and as a complement to the usual method in the final stage of irrigation and for investigating the effectiveness of laser in root canal disinfection, the 810 nm diode laser, based on the results of the present study, has the best result in terms of reducing the number of *E. faecalis* colonies, which is consistent with the results of other studies.<sup>21,36,44-46</sup> One of the reasons for the positive effect of the laser on *E. faecalis* colonies is the greater ability of the 810 nm diode laser to penetrate deep into the dentinal tubules.<sup>22,47</sup> In fact, due to the progressive reduction in the size of the dentine tubules by moving towards the apical areas which limit the penetration of irrigation materials, the inherent characteristics of the laser (including light scattering, local intensity increase, and beam attenuation) provide a way for deeper penetration of the radiation into dentine tubules. Further, its fine optic fiber allows increasing laser power and easy access to the apical third even in curved canals.<sup>7</sup> On the other hand, the small absorption coefficient of this wavelength in water allows more penetration of radiation, and this issue can be considered another reason for achieving better antibacterial effects.<sup>39</sup> Also, in the present study, the power of 1.5 W of this type of laser was used, which is considered an effective power in destroying *E. faecalis* in both clinical and laboratory conditions.<sup>3</sup>

On the other hand, to explain the method of the antibacterial effect of laser, it should be mentioned that the absorption of laser energy in the target tissue is accompanied by an increase in temperature. Laser rays cause photochemical changes in living cells. In addition, the effect of different types of lasers on biofilms is different based on their water content, extracellular matrix components, cell density, and absorption properties, and the anti-biofilm performance of the laser can be attributed to the amount of water absorption in the biofilm and the occurrence of thermal necrosis in the remaining living cells.<sup>1</sup> Furthermore, regarding the mechanism of the laser effect against *E. faecalis* bacteria, Moritz<sup>48</sup> observed that the destructive reaction between the ions emitted from the laser and the protein molecules of the cell wall eventually leads to the rupture of the bacterial membrane. This, even if in the smallest measure, causes major changes in the shape of bacteria. Laser thermal changes and temperature increases up to 42-52 °C can also be effective in destroying the cell membrane and creating biomolecular changes in this bacterium.<sup>31</sup>

In examining the effectiveness of the 980 nm diode laser in this study, it was found that its antibacterial ability was less than that of the 810 nm diode laser and the PUI method, but it was more effective than the GF group in all comparisons between test groups. However, in the two-way comparison between this laser and the ultrasonic group, no significant difference was found, which is consistent with the results of the study by Asnaashari et

al<sup>32</sup> and studies by Gutknecht et al.<sup>22,49</sup> From these results, it is inferred that the maximum absorption coefficient of the 980 nm diode laser in water prevents its deeper penetration into the dentinal tubules.<sup>39</sup> Also, higher energy radiations leave more destructive thermal effects than lower energy radiations.<sup>30</sup> In addition, since the wavelength and laser energy are inversely proportional, stronger antibacterial effects of the 810 nm laser can be expected.

There was no difference between the 980 nm diode laser and PUI. This indicated the same reduction effect of both methods. Of course, compared to the usual irrigation method, the effect of PUI was slightly better. In the study by Yang and Kim,<sup>50</sup> unlike the present study, the effect of the ultrasonic method was reported to be greater than that of the 980 nm diode laser; however, in the study by Elbatouty and Nour,<sup>1</sup> the 980 nm laser group was more effective. In Jambagi and colleagues' study,<sup>30</sup> the 940 nm diode laser was more effective than the continuous ultrasonic method and regular irrigation. In their study, Kaur et al<sup>26</sup> first found the PUI as the most effective method and in their study, the 810 nm diode laser and the conventional irrigation method were ranked next, respectively. Gründling et al<sup>51</sup> concluded in their study that PUI can be an effective adjunctive method in root canal disinfection. These contradictory results can be attributed to the different designs of the studies, including the number and type of samples, the volume and concentration of the irrigation materials used, the various preparation methods of the canals, the difference in the devices and their application methods, and the different settings and wavelengths of the lasers. All the three mentioned methods are effective in reducing the number of *E. faecalis* colonies. The PUI method increases the effect of sodium hypochlorite and EDTA by creating an acoustic flow and delivering the irrigation materials to all areas of the canal, even the areas that have not been prepared. Also, the shear stress created on the canal wall in this method leads to debris separation, individual microbes, and biofilms.<sup>1</sup> The disintegration of the biofilm also makes the resulting planktonic bacteria more sensitive to the antibacterial action of sodium hypochlorite.<sup>30</sup> The study by van der Sluis et al<sup>52</sup> showed that the protocol of using the ultrasonic method is affected by the taper of the file and the diameter of the root canals. Therefore, the higher the file tip, the more debris comes out of the canal. Other factors that affect the amount of debris removal are the irrigation time and the amount of detergent used.

In examining the effectiveness of the finishing brush of the Gentle File system, the results showed that this method is effective in reducing the number of *E. faecalis* colonies compared to the positive control group, but compared to the conventional irrigation group, it had the lowest reduction effect, which is consistent with the result of a study by Neelakantan et al.<sup>20</sup> In their study, it was found

that to achieve cleaner canals in single-rooted teeth, the methods of using tools and activating the cleaning agent and their speed are more effective than the size of the apical region in reducing the amount of remaining tissue. Also, the GF finisher brush, which plays its role in helping to clean the canal by opening its 6 wire strands while rotating and by scratching the walls, can remove biofilms and tissues attached to the canal wall more effectively than irrigation with a syringe. This is another reason for the superiority of this method over regular irrigation.<sup>20</sup> However, despite all the mentioned advantages, choosing this method among the test methods in the present study is not preferred over others due to the weaker results, and it is necessary to design and conduct more studies on its effectiveness in root canal disinfection.

## Conclusion

Compared to the positive control group that did not receive any irrigation and activation method, all test methods including 810 and 980 nm diode lasers, PUI, Gentle file finisher brush, and conventional irrigation were effective in reducing *E. faecalis* colonies. Also, it can be said that as a complementary method to conventional irrigation with a syringe, the first choice can be the 810 nm diode laser. In addition, regular irrigation alone does not have enough effect on bacteria that can initiate persistent or recurrent infections and treatment failure.

## Authors' Contribution

**Conceptualization:** Shiva Shirani lapari, Maryam Zare Jahromi.

**Data curation:** Maryam Zare Jahromi, Arezoo TahmoresPour.

**Formal analysis:** Shiva Shirani lapari, Amir Mansour Shirani.

**Funding acquisition:** Shiva Shirani lapari.

**Investigation:** Shiva Shirani lapari, Maryam Zare Jahromi.

**Methodology:** Maryam Zare Jahromi, Arezoo TahmoresPour, Amir Mansour Shirani.

**Project administration:** Maryam Zare Jahromi.

**Resources:** Shiva Shirani lapari.

**Software:** Arezoo TahmoresPour, Amir Mansour Shirani.

**Supervision:** Maryam Zare Jahromi, Arezoo TahmoresPour, Amir Mansour Shirani.

**Validation:** Maryam Zare Jahromi, Arezoo TahmoresPour, Amir Mansour Shirani.

**Writing—original draft:** Shiva Shirani lapari, Maryam Zare Jahromi.

**Writing—review & editing:** Shiva Shirani lapari, Maryam Zare Jahromi, Arezoo TahmoresPour, Amir Mansour Shirani.

## Competing Interests

None.

## Ethical Approval

This study was approved by the ethics committee of Islamic Azad University, Isfahan (Khorasgan) branch coded as IR.IAU.KHUISF.REC.1400.015

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