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Effectiveness of Calcium Hydroxide Against Enterococcus Faecalis Biofilm in Dental Root Canals

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ABSTRACT

Enterococcus faecalis (E. faecalis) is the most widely detected species associated with persistent endodontic infections because it has the ability to form biofilms. This study aimed to identify the effectiveness of calcium hydroxide paste Calcigel® (Prevest DenPro, India) on E. faecalis (ATCC 29212) biofilms in root canals with exposure times of 14, 21, and 28 days. The type of research used was true experimental in vitro by randomly selecting samples of tooth roots on the 14th day of biofilm development and dividing them into six groups: use of Ca(OH)2 as intra-canal medicament for 14, 21, and 28 days, as well as positive control without treatment for 14, 21, and 28 days. Dentin chip suspensions were used for colony form ng units counting to estimates remaining E. faecalis counts. The effectiveness calculation obtained by comparing the test group with the control group at the same treatment duration, with the results of using Ca(OH)2 for 14 days showing a decrease in the number of E. faecalis 96.46%, the results of using Ca(OH)2 for 21 days was 95.20%, while the results of using Ca(OH)2 for 28 days was 94.59%. The results of testing the hypothesis with the ANOVA test showed that there was no significant difference between the effectiveness of calcium hydroxide with exposure to 14 days, 21 days, and 28 days with a significance value of <0.452.

INTRODUCTION

Pulp and periapical disease is one of the diseases that has a high prevalence in the field of dentistry in Indonesia. The results of data from the Ministry of Health in the Basic Tabulation List (DTD) in Azzuhdi (2021), show that pulp and periapical disease in Indonesia ranks 7th in the top 10 diseases in outpatients in all hospitals in Indonesia with a total of 86,421 cases with details of 46,994 cases in female patients and 39,427 cases in male patients. (Azzuhdi, M. Erlita, I. Azizzah, 2021)Treatment to handle cases of pulp and periapical disease is endodontic treatment. The goal of endodontic treatment is thorough *debridement* and cleaning of the root canal system from any infected pulp tissue so that the root canal space can be formed and prepared to be filled with *inert materials* to prevent or minimize the possibility of re-infection. (Prayogo et al., 2019)Endodontic treatment can be divided into three stages (*endodontic triad*), namely biomechanical preparation of the root canal (*cleaning and shaping*), sterilization, and root canal filling. (Novitasari & Nugroho, 2017)

Several studies have evaluated the success or failure of endodontic therapy and reported success rates ranging from 40% to 93%. This wide range is likely due to differences in clinical procedures, experimental designs, evaluation criteria, and length of observation periods. (Srinivasan & Raghu, 2016)The success of endodontic treatment is influenced by the ability to eliminate microorganisms in the infected root canal, such as the use of intracanal medicaments. The purpose of using intracanal medicaments is to eliminate bacteria remaining in the root canal that cannot be removed by chemomechanical preparation, reduce periradicular inflammation and reduce pain, prevent or stop inflammatory root resorption, and prevent re-infection of the root canal. (Permatasari & Irbahani, 2021)

Enterococcus faecalis (E. faecalis) is the most frequently detected enterobacterial species in endodontic-related infections and is sometimes the only isolate found in root canals. The prevalence of *E. faecalis* detected in persistent endodontic infections varies widely, ranging from 24 to 77%. (Bachtiar et al., 2015; Saffari et al., 2018) *E. faecalis* has several virulence factors, one of which is the ability to form biofilms so that *E. faecalis* can survive even though chemomechanical processes and medicament administration have been carried out. (Bachtiar et al., 2015; Saffari et al., 2018)*E. faecalis* biofilms is adjusted to three main

stages: (1) bacterial attachment and microcolony formation, (2) dissolution of mineralized dentin substrate and release of calcium and phosphate ions, and (3) development of mineralization and calcification in the biofilm. As the biofilm matures in the root canal space, the *E. faecalis biofilm* becomes calcified, resulting in persistent root canal infections. (Zand et al., 2014)Du *et al* . observed dentin sections incubated with *E. faecalis* and found young biofilms on day 1 and mature biofilms on day 21. (Du et al., 2014)

Calcium hydroxide is a popular intracanal medicament and is considered *the gold standard* because it has broad-spectrum antibacterial properties, is biocompatible with tissue, reduces periapical tissue inflammation, and can stimulate the formation of hard tissue used to eliminate residual microorganisms after chemomechanical preparation. (Ariani & Hadriyanto, 2013; Kim & Kim, 2015)The average time of endodontic treatment using calcium hydroxide is 1-4 weeks. (Trilaksana, 2013)Andrade *et al* . stated that it takes at least 14 days to increase the penetration of calcium hydroxide paste so that it can directly contact bacteria that can penetrate into the dentinal tubules such as *E. faecalis* . (de Andrade et al., 2021)Baker *et al* . reported that calcium hydroxide has poor antibacterial activity against *E. faecalis* . (Ghatole et al., 2016)This study was conducted to identify the effectiveness of calcium hydroxide paste preparation Calcigel® (Prevest DenPro, India) against *E. faecalis biofilm* in root canals with a treatment time span of 14, 21, and 28 days, because there has been no published research on PubMed® with the materials and treatment time as described, because generally the studies conducted focus on using other types of calcium hydroxide paste preparations such as Vitapex®, Metapex®, and UltraCal™ (T. Panyakorn *et al.* , 2021; BM Marín-Correa *et al.* , 2020; Latham *et al.* , 2016; Kim D and Kim E., 2015) and do not compare the application time of calcium hydroxide Calcigel® on days 14, 21, and 28 (M. Selvi *et al.* , 2022), so the authors are interested in studying this. (Kim & Kim, 2015; Latham et al., 2016; Marín-Correa et al., 2020; Panyakorn et al., 2021; Selvi et al., 2022)

Several studies have explored the antimicrobial efficacy of calcium hydroxide against *Enterococcus faecalis* biofilms in dental root canals, recognizing its widespread use as an intracanal medicament. Research by Kim and Kim (2015) emphasized the limitations of calcium hydroxide in eliminating *E. faecalis* biofilms due to the bacterium's ability to survive in high pH environments, which often reduces the medicament's effectiveness over time. Similarly, Momenijavid et al. (2022) demonstrated that the biofilm structure of *E. faecalis* becomes denser with prolonged exposure to calcium hydroxide, reducing its antimicrobial action. Du et al. (2014) reported that long-term exposure to endodontic disinfectants like calcium hydroxide could lead to adaptive changes in *E. faecalis* biofilms, increasing their resistance. Meanwhile, studies by Panyakorn et al. (2021) and Marín-Correa et al. (2020) have focused on comparing different calcium hydroxide formulations, such as Vitapex® and Metapex®, to determine their efficacy against *E. faecalis*, but there is limited research on the effectiveness of Calcigel® over varying exposure times.

Despite extensive studies on calcium hydroxide's antimicrobial properties, there remains a lack of research evaluating the effectiveness of Calcigel® specifically against *E. faecalis* biofilms over different exposure durations (14, 21, and 28 days). Previous studies primarily focused on other formulations and did not comprehensively assess the impact of prolonged application times on biofilm eradication. Additionally, while many studies have examined the antibacterial action of calcium hydroxide in general, there is minimal focus on its long-term effects on biofilm structure and bacterial survival in the context of Calcigel®. This gap in the literature highlights the need for a more in-depth analysis of how exposure duration influences the efficacy of Calcigel® against *E. faecalis* biofilms, especially given the bacterium's resistance mechanisms.

This study presents a novel approach by specifically evaluating the antimicrobial efficacy of Calcigel® (Prevest DenPro, India) against *E. faecalis* biofilms over 14, 21, and 28-day exposure periods. Unlike previous research, which focused on other calcium hydroxide formulations, this study examines the time-dependent effectiveness of Calcigel®, providing insights into its optimal application duration. The study also contributes to the understanding of how prolonged exposure to calcium hydroxide affects biofilm integrity and bacterial survival, offering valuable data for endodontic treatment strategies targeting persistent *E. faecalis* infections.

The primary objective of this study is to assess the effectiveness of Calcigel® against *E. faecalis* biofilms in dental root canals over varying exposure durations (14, 21, and 28 days) to determine the optimal treatment period for maximum antimicrobial efficacy. The findings aim to provide dental practitioners with evidence-based guidance on the use of Calcigel® as an intracanal medicament, potentially improving clinical outcomes in endodontic treatments. Furthermore, the study's insights into the interaction between biofilm structure and exposure time can inform future research on enhancing endodontic disinfection protocols, ultimately contributing to better patient care and reduced rates of treatment failure due to persistent infections.

METHOD

The type of research used was a pure *in vitro experiment* by randomly selecting tooth root samples on the 14th day of biofilm development and dividing them into six groups with the following divisions: use of intracanal medicaments Calcigel® (Prevest DenPro, India) for 14, 21, and 28 days, and a positive control without treatment for 14, 21, and 28 days. *Dentin chips suspension* was used for colony forming unit (CFU) counting to estimate the number of remaining *E. faecalis*. The study was conducted from May to June 2023 at the Central Laboratory of Padjadjaran University. This study has received ethical clearance from the Research Ethics Commission of Padjadjaran University (335/UN6.KEP/EC/2023).

The population in this study was bacteria of the *Enterococcus genus* with a research sample of *E. faecalis* ATCC 29212 available at the Central Lab, Unpad. Pure culture of *E. faecalis* was prepared in *brain-heart infusion broth* (BHIB) (Lab M, Bury, United Kingdom) and incubated for 24 hours at 37 ◦ C. Bacterial suspension was made by centrifugation and suspended. The bacterial suspension used was equivalent to a standard solution of 0.5 McFarland. (Zand et al., 2014)

All calculus and periodontal tissue on the teeth were removed with *an ultrasonic device* (*scaler*). The central incisor tooth samples were opened for cavity access (open cavity) using *an endo access bur* (Dentsply Maillefer, Ballaigues, Switzerland), the pulp chamber was cleaned and the orifice was obtained. The working length of each central incisor tooth sample was measured using a digital caliper (*Digital Caliper*). (Pladisai et al., 2016; Zand et al., 2014)The central incisor tooth samples were then extirpated with pulp tissue with an extirpation needle and *cleaned and shaped with the crown down* technique using *ProTaper* (Dentsply Maillefer, Ballaigues, Switzerland). Irrigation was performed during instrumentation with a 5.25% NaOCl solution using a 2 mL *syringe* and a 30 *gauge needle. After instrumentation was complete, the root canal was rinsed with 1% ethylenediaminetetraacetic acid* (EDTA) solution and *phosphatebuffered saline* (PBS). The crown of the tooth was removed (*decoronated*) with *carbondurum discs* near the CEJ leaving ~12 mm of root length. (Zand et al., 2014)

Biofilm verification was performed by incubating one root specimen into sterile BHIB (Lab M, Bury, United Kingdom) as a sterile control for 28 days and three root specimens were immersed in 5 mL of *E. faecalis culture* (*optical density* = 0.5) in BHIB (Lab M, Bury, United Kingdom) and incubated for 14, 21, and 28 days at 37 ◦ C as negative controls, respectively. The bacterial suspension was replaced with fresh media 3 times a week and periodically checked for contamination by *Gram staining* and replating on *Mueller-Hinton agar* (Merck, Germany). (Pladisai et al., 2016)After the incubation period, 4 specimens were gently washed with 1% *phosphate buffered saline* (PBS). The specimens were sectioned lengthwise, dried, and examined using *a Tabletop Microscope* at a magnification of 1000 - 2500 times. (Figure 1) (Pladisai et al., 2016; Zand et al., 2014)

The tooth root samples for the test and control groups were autoclaved at 121 ◦ C 15 Psi for 20 minutes, to kill all microorganisms. All teeth were stored in BHIB media (Lab M, Bury, United Kingdom) at 37 ◦ C for 24 hours to see the effectiveness of the sterilization process. Each root in a sterile tube (*falcon tube*) containing BHIB media (Lab M, Bury, United Kingdom) was added with 2 mL of standard suspension *of E. faecalis* . Nutrients were added daily to ensure *nutritional support* and media stability by replacing

BHIB media (Lab M, Bury, United Kingdom) every 24 hours, and the temperature was maintained at 37 °C. (Zand et al., 2014)

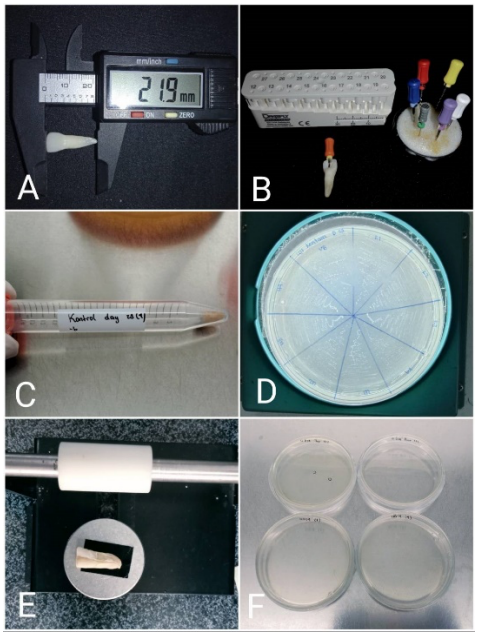
On the 14th day of biofilm development, roots were randomly selected and divided into six groups with the following division:

Table 1 Distribution of treatment groups of tooth root samples

Treatment	N	Time		
		14 Days	21 Days	28 Days
Test	12	4 Root canal samples were filled with $Ca(OH)_2$ Calcigel® paste preparation (Prevest DenPro, India) and incubated at 37 °C for 14 days.	4 Root canal samples were filled with $Ca(OH)_2$ Calcigel® paste preparation (Prevest DenPro, India) and incubated at 37 °C for 21 days.	4 Root canal samples were filled with $Ca(OH)_2$ Calcigel® paste preparation (Prevest DenPro, India) and incubated at 37 °C for 28 days.
Control	12	4 Root canal samples were not treated, only incubated for 14 days at 37 °C.	4 Root canal samples were not treated, only incubated for 21 days at 37 °C.	4 Root canal samples were not treated, only incubated for 28 days at 37 °C.

After the treatment period (14 days, 21 days, and 28 days), all tooth root samples were cleaned using sterile *paper points.* and saline solution. A thin layer of the *internal surface of the root canal* in the middle 1/3 of the root was taken with *Gates Glidden drills* #6, so that 10 mg of *dentin chips were obtained for each sample, then the dentin chips* were put into a sterile tube, 2 mL of physiological solution was added and homogenized for 20 seconds. Ten-fold serial dilution was carried out to a concentration of 10^{-8} as a sample solution. 100 µL of the sample solution was spread using the *spread plate technique on Mueller-Hinton* agar plates (Merck, Germany) and incubated at 37 °C for 24 hours. The number of colony forming units (*CFU*) per milliliter was counted on each *Mueller-Hinton agar plate* (Merck, Germany) using *colony counter*. (Zand et al., 2014)

Figure 1Determination of working length of tooth sample (A). *Crown-down technique equipment* (B). One tooth root sample in a sterile *falcon tube* (C). Contamination test results (D). Placement of root sample for TM 3000 (E). TPC test results *Day*- 14 (F)



The null hypothesis proposed in this study is that there is no significant difference. between the effectiveness of calcium hydroxide with exposure of 14 days, 21 days, and 28 days. Data were analyzed using *Statistical Package for Social Science (SPSS*) software. The *Shapiro-Wilk normality test* was conducted to determine whether the data was normally distributed or not. The homogeneity test was conducted to determine the variance of the data population whether two or more groups of data had the same or

different variances as a prerequisite in the analysis of variance test. The results of the *Shapiro-Wilk normality test* showed normally distributed data results and the results of the *Levene homogeneity test* showed homogeneous data results, so the *one way analysis of variance* (ANOVA) test was used to test the difference in bacterial reduction between the 6 groups. The results of the ANOVA test showed a significant difference, so a further ANOVA (*Post-Hoc Test*) test was conducted to see which groups were significantly different. The comparison between the test group and the control group with the same exposure time was used in calculating the percentage of calcium hydroxide effectiveness, using the calculation method: (Asnaashari et al., 2022; Nuryadi et al., 2017; Pladisai et al., 2016; Purnomo, 2016)

$$\%Efektivitas = \frac{\overline{CFU\ Kontrol} - \overline{CFU\ Uji}}{\overline{CFU\ Kontrol}}\ x\ 100$$

Results

calculation was obtained by comparing the test group with the control group at the same treatment time, with the results of using Ca(OH) 2 for 14 days showing a decrease in the number of *E. faecalis* of 96.46% , using Ca(OH) 2 for 21 days of **95.20%** , and using Ca(OH) 2 for 28 days of 94.59%.

Table 1TPC results and percentage of calcium hydroxide effectiveness based on exposure time.

Time	Treatment	N	Cup 1	Cup 2	Cup 3	Cup 4	Average (CFU/mL)	% of surviving bacteria	% Effectiveness of Ca(OH) 2
14 Days	Test	4	8	4	11	7	7.50	3.54%	96.46%
	Control	4	237	140	282	188	211.75		
21 Days	Test	4	15	14	10	8	11.75	4.80%	95.20%
	Control	4	258	266	302	154	245.00		
28 Days	Test	4	17	21	20	15	18.25	5.41%	94.59%
	Control	4	300	400	350	300	337.50		

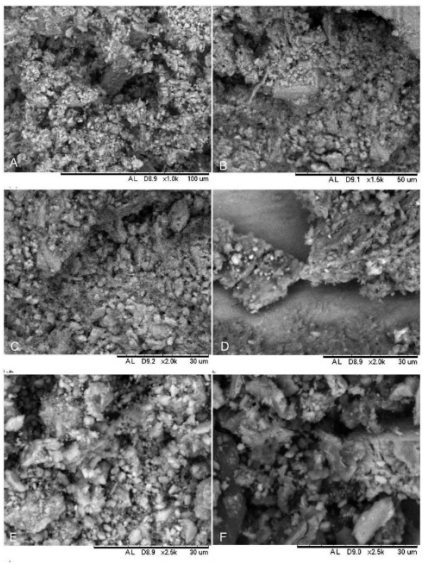
The effect of intracanal calcium hydroxide medicament Calcigel® paste (Prevest DenPro, India) on the reduction of *E. faecalis counts* at various exposure times is presented in Table 3, which depicts the means, ranges, and standard deviations observed in the test groups. The results of *one-way analysis of variance* (ANOVA) showed no significant difference in bacterial counts between the three test groups (p = 0.452, df = 9).

Table 2One Way Analysis of Variance (ANOVA) Test Data Presentation Table

Treatment	Time	N	Average (CFU/mL)	Min.	Max.	Standard Deviation	Sig.	Conclusion
Test	14 days	4	7.50	4	11	2,887	0.452	There was no significant difference between the effectiveness of calcium hydroxide with 14-day, 21-day, and 28-day exposure.
	21 days	4	11.75	8	15	3.304		
	28 days	4	18.25	15	21	2,754		

Results of observations of tooth root canals using *a Tabletop Microscope* (TM 3000) showed that *E. faecalis biofilm* had grown on days 14, 21, and 28 (Figure 2).

Figure 2*Enterococcus faecalis* biofilm aged 28 days (A and B), 21 days (C and D), and 14 days (E and F) in the root canal using *a Tabletop Microscope* 3000 at a magnification of 1000x - 2500x.



Discussion

Calcium hydroxide is recommended as an intracanal medicament because it is antibacterial, soluble in tissue, can prevent tooth resorption, control exudate in teeth with persistent periapical inflammation and stimulate tissue repair with hard tissue formation. The role of calcium hydroxide as an intracanal medicament is to stimulate antibacterial activity, eliminate carbon dioxide used by anaerobic bacteria for respiration, cause damage to the bacterial cytoplasmic membrane, protein denaturation and DNA damage. (Trilaksana, 2013)Calcium hydroxide provides antimicrobial activity by dissociating into calcium and hydroxyl ions. Calcium and hydroxyl ions are highly oxidant anions and show extreme reactivity with several biomolecules. The main effect of calcium hydroxide is associated with the action of anions that can increase pH. The alkaline environment detoxifies bacterial lipopolysaccharides (LPS) by eliminating esterified fatty acids and changing their chemical conformation, resulting in the integrity of the bacterial cytoplasmic membrane being destroyed. Hydroxyl ions exert antimicrobial effects after diffusion through the entire root canal system through direct or indirect contact. (Cosan et al., 2022)

The pH level of calcium hydroxide paste depends on the ratio of calcium hydroxide to its solvent, a high ratio of calcium hydroxide powder can produce a pH > 11. The recommended pH level of calcium hydroxide paste preparation ranges from 8.6-10.3, because pH > 11 can cause cytotoxicity in the periapical area. Histopathological analysis showed evidence of better apical and periapical tissue repair with the formation of a *mineralized apical barrier* in specimens with a treatment time of 15 and 30 days, and showed poor results in specimens with a treatment time of 7 days. (Pedrinha et al., 2022)The results of the studies of Chany *et al.,* and Sjögren *et al* . stated that calcium hydroxide efficiently eliminates microorganisms and provides predictable results when inserted into the root canal for 7 days. (Putri et al., 2022), (Ordinola-Zapata et al., 2022)In many in vitro studies, it has been reported that the use of calcium hydroxide for 5 weeks or more causes a decrease in root fracture resistance, so the use of calcium hydroxide exceeding one month should be used with caution. (Lee, 2013)

E. faecalis is a non-spore-forming bacterium, facultative anaerobic because it has the ability to grow in the presence or absence of oxygen. (García-Solache & Rice, 2019; Xuedong & Yuqing, 2015) *E. faecalis* can survive in environments with poor nutrient supply and high alkaline pH up to 11.5. (Alghamdi & Shakir, 2020) *E. faecalis* can grow optimally at temperatures of 35°C to 37°C and can survive at temperatures between 10 ^{o} C – 45 ^{o} C. (García-Solache & Rice, 2019) *E. faecalis* is one of the bacteria associated with persistent root canal infections, indicating that *E. faecalis* has the capacity to survive chemomechanical procedures and environments with limited nutrients. Specific survival mechanisms that may be used to survive alkaline stress include, (1) activation of the ion transport system to balance intercellular and external pH levels (*proton pump inhibitor mechanism*), (2) intrinsic resistance, (3) neutralization of medicaments by bacterial cells, and (4) changes in gene expression to changes in environmental conditions. (Cathro et al., 2022)The results of the study by Cathro, *et al* (2022) showed that membrane proteins are involved in the formation of protective capsules/ *Extracellular polymeric substances* (EPS) that protect cells from damaging OH^- ions. The production of this EPS is facilitated by increased polysaccharide biosynthesis. The role of membrane proteins in this coordinated response to increased pH may help explain cell adaptation to the extreme alkaline pH of calcium hydroxide. (Cathro et al., 2022)

The ability of *E. faecalis* to grow as a biofilm on the root canal wall and as a monoinfection in the treated canal without synergistic support from other bacteria makes its high resistance to antimicrobial

agents a pathogen that is very resistant to root canal treatment. (Alghamdi & Shakir, 2020)Microbial cells in biofilms have shown antibiotic resistance 10-1000 times stronger than planktonic cells. (Divakar et al., 2019)*E. faecalis* biofilms can be eliminated with calcium hydroxide at pH 12. (Pedrinha et al., 2022)In the study of Bulacio *et al* ., *E. faecalis* isolated from root canals can produce biofilms on *microplates* since day 14 and the biofilm has matured *on* day 30. (Bulacio et al., 2015)The results of the study by Guerreiro-Tanomaru *et al* . also showed similar results, namely on days 14 and 21. (Guerreiro-Tanomaru et al., 2013)

The results of the research that has been done show that the effectiveness of calcium hydroxide decreases with increasing exposure time, where optimal results are obtained in calcium hydroxide with an exposure time of 14 days. This is in line with research conducted by Momenijavid et al. which shows that the components of calcium hydroxide, namely Ca $^{2+}$ ions, and alkaline pH, cooperatively strengthen the biofilm, so that calcium hydroxide does not eradicate *E. faecalis biofilm*, but encourages biofilm growth. (Momenijavid et al., 2022)$^{2+}$ ions causes the biofilm to be denser with more cavities and indicates an increase in EPS. The presence of Ca $^{2+}$ ions also creates a granular surface on the biofilm, biomass enlargement and increased thickness, colony size, and biofilm volume. The alkaline pH of the environment increases the absorption of Ca $^{2+}$ when the biofilm is treated with Ca $(OH)_2$; thus, alkaline pH is also able to affect the morphology, structure, and chemical properties of *E. faecalis biofilm*. (Momenijavid et al., 2022; Safari et al., 2014)

The results of the comparison between test groups in the study showed that there was no significant difference in effectiveness between the use of calcium hydroxide with a treatment time of 14 days (average CFU/mL = 7.50), 21 days (average CFU/mL = 11.75), and 28 days (average CFU/mL = 18.25) with a significance value of 0.452. The results of the study by Asnaashari *et al.,* showed that calcium hydroxide could not reduce the number of 21-day-old *E. faecalis biofilms* from the root canal. (Asnaashari et al., 2022)The results of the study by Latham *et al.,* showed that calcium hydroxide was unable to completely remove *E. faecalis* from artificial necrotic permanent teeth. (Latham et al., 2016)Research by Reyhani *et al.,* which tested the effectiveness of calcium hydroxide in root canals with 4-week (*immature*) and 6-week (*mature*) biofilms showed that the antimicrobial properties of nano-calcium hydroxide were higher than conventional calcium hydroxide in *mature biofilms* , and had no significant difference in effectiveness in *immature biofilms* . (Frough-Reyhani et al., 2016)The results of the study by Eskandarinezhad *et al.,* showed that the percentage of effectiveness of calcium hydroxide against *mature biofilms* (6 weeks old) was 99.41% in root canals. (Eskandarinezhad et al., 2022)Based on the studies mentioned, it can be concluded that *E.* faecalis cannot be completely eliminated with calcium hydroxide because *E. faecalis* has *a proton pump inhibitor mechanism* to balance pH. (Cathro et al., 2022; Eskandarinezhad et al., 2022)

The effectiveness of intracanal medicaments can be influenced by the preparation technique used. According to Schilder, a good root canal *cleaning and shaping technique mechanically meets the following criteria: (1) The root canal preparation must form a continuously tapering funnel* from the root apex to the coronal cavity access, (2) The *cross-sectional diameter* of the preparation must be narrower towards the apically and wider as it approaches the cavity access, (3) The root canal preparation must follow the shape of the original root canal, (4) The apical foramen must remain in its original spatial relationship to the bone and root surface, (5) The apical opening must be made as small as possible in all cases. Good biological root canal techniques include, (1) Limit instrumentation to the root canal, (2) Be careful to apply pressure to necrotic material so that it does not come out of the apical foramen, (3) Remove all tissue debris from the root canal system, (4) Complete the *cleaning and shaping process* in one visit, and (5) Create enough space during root canal enlargement to accommodate intracanal medicaments and potential exudate reception. (Neelakantan et al., 2022)The results of good *cleaning and shaping will produce ' glassy smooth* ' root canal walls and no debris or irrigation material remaining in the root canal. (Singla et al., 2021)

Placement of intracanal medicament can also affect the effectiveness of calcium hydroxide in eliminating *E. faecalis*. The results of the study by Tan., *et al* showed that the use of a specially designed *paste carrier* was more effective than the use of *a syringe* and *finger spreader #25* (group 1), *syringe* and *rotary lentulo spiral #4* (group 2) in the placement of calcium hydroxide. (Tan et al., 2013)In the study conducted, ready-to-use calcium hydroxide paste Calcigel® (Prevest DenPro, India) was used with placement in accordance with the manufacturer's instructions, namely (1) Install a disposable applicator on *the syringe* , (2) Insert the calcium hydroxide paste into the clean root canal, (3) Allow to dry.

Some limitations found in this study are the unavailability of *Scanning Electron Micrograph* (SEM), so that the image of *E. faecalis biofilm is less visible* and the dentin tubules cannot be observed in tooth root samples using *Tabletop Microscope* (TM 3000). *Second, the* very small size of *E. faecalis colonies (small colony variants*) makes it difficult to count colonies.

Suggestions for further research: *in vivo research can be conducted* to directly determine the effectiveness. various intracanal calcium hydroxide medicaments against *E. faecalis* biofilm with *a* recommended interappointment time of 14 days. Research can also be conducted on the effectiveness of calcium hydroxide on various ages of *E. faecalis biofilm development* considering that the more mature the biofilm age can cause bacteria to become more resistant. Other research that can be conducted is on the effectiveness of calcium hydroxide with the addition of various other medicaments at different exposure times in the root canal.

CONCLUSION

The results of hypothesis testing using the ANOVA test showed that there was no significant difference between the effectiveness of calcium hydroxide with exposure of 14 days, 21 days, and 28 days with a significance value of <0.452.

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