**BUKTI REVIEW JURNAL**

Original Article

# Efficacy of Bioceramic and Calcium Hydroxide-Based Root Canal Sealers against Pathogenic Endodontic Biofilms: An *In vitro* Study


**Abstract**

**Background:** Complete eradication of root canal pathogens cannot be predictably achieved by chemomechanical preparation and root canal disinfection. Therefore, an obturation material that has superior antimicrobial activity and sealing ability is required to inactivate residual microbes and prevent them from reentering the root canal system. Recently developed bioceramic root canal sealers are hydraulic cement which form calcium hydroxide during the hydration process. Like calcium hydroxide sealers, they exert an antimicrobial effect by releasing hydroxyl ions and increasing the pH. **Objective:** The objective of this study was to evaluate and compare the antimicrobial activity of a calcium hydroxide-based sealer and two bioceramic sealers against *Porphyromonas gingivalis*, *Enterococcus faecalis*, and *Candida albicans* biofilms. **Materials and Methods:** The sealers were dissolved in sterile saline to obtain supernatants. Biofilm formation assays, colony counting, and real-time polymerase chain reaction (PCR) were performed to evaluate the antimicrobial activity of each supernatant. The data were analyzed using one-way analysis of variance. **Results:** All sealers exerted effects against all three microbial biofilms. The biofilm formation assays showed that the bioceramic sealers were more effective against *P. gingivalis* and *E. faecalis* biofilms. In contrast, colony counting and real-time PCR showed that the calcium hydroxide sealer was significantly more effective than the bioceramic sealers. All tests showed that the calcium hydroxide sealer was more effective against *C. albicans*, with the colony count and real-time PCR results showing statistically significant differences. **Conclusion:** The calcium hydroxide-based sealer was more effective than the bioceramic sealers in eradicating pathogenic root canal biofilms.



**Keywords:** *Antimicrobial activity, bioceramic sealer, calcium hydroxide-based root canal sealer, Candida albicans, Enterococcus faecalis, Porphyromonas gingivalis*



**Tien Suwartini[1], Jessica Santoso[2], Armelia Sari Widyarman[3], Dina Ratnasari[1]**

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**Tien Suwartini <tien.s@trisakti.ac.id>**

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1 message

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| | • Brain heart infusion broth normally helps in proliferation of bacteria. Hence buffered phosphate buffer is preferred rather than BHI | Brain heart infusion broth was used in this study as the culture media, in accordance with previous studies. Buffered phosphate buffer or PBS was used to wash microtiter plate before crystal violet assay. | No changes |
| | • This type of evaluation does not simulate an in vivo or clinical situation because the oral cavity bacteria are presented in a biofilm form, which guarantee a protected mode of growth in a hostile environment. Further there are other factors as pH modulation capabilities of dentin. Pure laboratory studies when compared with dentin model design have shown contradictory results. So clinical translational is questionable. How would you justify your study? | Pure laboratory studies were done to examine the ability of the sealers to eradicate oral pathogens which were often found inside the root canal and periapical tissue; without being affected by root canal anatomy, biomechanical preparation, root canal irrigation and disinfection, etc.<br><br>Further clinical studies should be done to examine and compare the sealers' antimicrobial effect in human teeth *in vitro*, or *in vivo* if possible, as hostile environment inside the root canal along with other factors/variables may affect the final result differently. | Page 12, Line 10 |

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| | • Provide reference for Pathogen culture procedures | Pathogen culture procedures were based on the Clinical and Laboratory Standards Institute. | Page 5, Line 3. Reference 32. |
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| | | | | | "Efficacy of Bioceramic and Calcium Hydroxide–Based Root Canal Sealers Against Pathogenic Endodontic Biofilms: An *In Vitro* Research" |
| 1 | Right | | 12 | [1]*Department of Conservative Dentistry, Faculty of Dentistry, Trisakti University, West Jakarta, Java, Indonesia,* | [1]*Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia,* |
| 1 | Right | | 15 | [2]*???, Postgraduate Program of Conservative Dentistry, Faculty of Dentistry, Trisakti University, West Jakarta, Java, Indonesia* | [2]*Conservative Dentistry Postgraduate Program, Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia* |
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| 3 | Right | 3 | 36 | One-way ANOVA test was performed, followed by Tukey's honestly significant difference *post hoc* test were used to determine the | One-way ANOVA test was performed, followed by Tukey's honestly significant difference *post hoc* test to determine the significance of |

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| | | | | significance of the differences between experimental groups. | the differences between experimental groups. |
| 3 | Right | 3 | 41 | The statistical analysis was performed using IBM SPSS® Statistics version 25 (IBM, USA). | The statistical analysis was performed using IBM® SPSS® Statistics 25.0 Desktop for Windows (IBM Corporation, New York, USA). |
| 7 | Right | | 15 | 13. Šimundić Munitić M, Poklepović Peričić T, Utrobičić A, Bago I, Puljak L. Antimicrobial efficacy of commercially available endodontic bioceramic root canal sealers: A systematic review. PLoS One 2019;14:e0223575 | 13. Šimundić Munitić M, Poklepović Peričić T, Utrobičić A, Bago I, Puljak L. Antimicrobial efficacy of commercially available endodontic bioceramic root canal sealers: A systematic review. PLoS One 2019;14(10):1-20. |
| 7 | Right | | 35 | 19. American Association of Endodontists. Guide to Clinical Endodontics. 6th ed. ???: ???; ???. Available from: https://www.aae.org/specialty/clinical-resources/guide-clinical-endodontics. [Last accessed on 2019 Nov 11]. | 19. American Association of Endodontists. Guide to Clinical Endodontics. 6th ed. Chicago: American Association of Endodontists; 2013. Available from: https://www.aae.org/specialty/clinical-resources/guide-clinical-endodontics. [Last accessed on 2019 Nov 11]. |
| 8 | Left | | 19 | 34. Trott A. Wound cleansing and irrigation. In: Wounds and Laceration. 4th ed., Ch. 7. ???: Elsevier Saunders; 2012. p. 73-4. | 34. Trott A. Wounds and Laceration. 4th ed. Philadelphia: Elsevier Saunders; 2012. Chapter 7, Wound cleansing and irrigation. p. 73-4. |
| 8 | Left | | 25 | 36. Miglani R, Shankar, Indira R, Ramachandran S. An *in vitro* evaluation of calcium hydroxide root canal sealers and its effect on six microorganisms. J Conserv Dent 2007;10:99-103. | 36. Miglani R, Shankar, Indira R, Ramachandran S. An *in vitro* evaluation of calcium hydroxide root canal sealers and its effect on six microorganisms. J Conserv Dent 2007;10:99-103. |

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| | | | | | (Note: the original article and citation doesn't provide the author's first name or initial) |
| 8 | Left | | 44 | 41. Rezende GC, Massunari L, Queiroz IO, Gomes Filho JE, Jacinto RC, Lodi CS, *et al.* Antimicrobial action of calcium hydroxide-based endodontic sealers after setting, against *E. faecalis* biofilm. Braz Oral Res 2016;30 :???. | 41. Rezende GC, Massunari L, Queiroz IO, Gomes Filho JE, Jacinto RC, Lodi CS, *et al.* Antimicrobial action of calcium hydroxide-based endodontic sealers after setting, against *E. faecalis* biofilm. Braz Oral Res 2016;30(1):1-6. |
| 8 | Right | | 8 | 46. Weckwerth PH, Lima FL, Greatti VR, Duarte MA, Vivan RR. Effects of the association of antifungal drugs on the antimicrobial action of endodontic sealers. Braz Oral Res 2015;29 :???. | 46. Weckwerth PH, Lima FL, Greatti VR, Duarte MA, Vivan RR. Effects of the association of antifungal drugs on the antimicrobial action of endodontic sealers. Braz Oral Res 2015;29(1) :1-7. |
| 8 | Right | | 14 | 48. Eldesouky HE, Mayhoub A, Hazbun TR, Seleem MN. Reversal of azole resistance in *Candida albicans* by sulfa antibacterial drugs. Antimicrob Agents Chemother 2018;62 :???. | 48. Eldesouky HE, Mayhoub A, Hazbun TR, Seleem MN. Reversal of azole resistance in *Candida albicans* by sulfa antibacterial drugs. Antimicrob Agents Chemother 2018;62(3):1-12. |
| 8 | Right | | 22 | 51. Marek C, Timmons S. Antimicrobials in pediatric dentistry. In: Nowak A, editor. Pediatric Dentistry: Infancy through Adolescence. 6th ed. ???: Elsevier; 2019. p. 128-41. | 51. Marek C, Timmons S. Antimicrobials in pediatric dentistry. In: Nowak A, editor. Pediatric Dentistry: Infancy through Adolescence. 6th ed. Philadelphia: Elsevier; 2019. p. 128-41. |
| 8 | Right | | 35 | 55. Hoshino RA, Silva GF, Delfino MM, Guerreiro-Tanomaru JM, Tanomaru-Filho M, Sasso-Cerri E, *et* | 55. Hoshino RA, Silva GF, Delfino MM, Guerreiro-Tanomaru JM, Tanomaru-Filho M, Sasso-Cerri E, *et al.* Physical |

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| | | | | *al.* Physical properties, antimicrobial activity and *in vivo* tissue response to apexit plus. Materials (Basel) 2020;13 :???. | properties, antimicrobial activity and *in vivo* tissue response to apexit plus. Materials (Basel) 2020;13(5):1-17. |
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AQ2

Original Article

# AQ3 Efficacy of Bioceramic and Calcium Hydroxide-Based Root Canal Sealers against Pathogenic Endodontic Biofilms: An *In vitro* Study

**Tien Suwartini[1], Jessica Santoso[2], Armelia Sari Widyarman[3], Dina Ratnasari[1]**

*[1]Department of Conservative Dentistry, Faculty of Dentistry, Trisakti University, West Jakarta, Java, Indonesia, [2]???, Postgraduate Program of Conservative Dentistry, Faculty of Dentistry, Trisakti University, West Jakarta, Java, Indonesia, [3]Department of Microbiology, Division of Oral Biology, Faculty of Dentistry, Trisakti University, West Jakarta, Java, Indonesia*

AQ4

## Abstract

**Background:** Complete eradication of root canal pathogens cannot be predictably achieved by chemomechanical preparation and root canal disinfection. Therefore, an obturation material that has superior antimicrobial activity and sealing ability is required to inactivate residual microbes and prevent them from reentering the root canal system. Recently developed bioceramic root canal sealers are hydraulic cement which form calcium hydroxide during the hydration process. Like calcium hydroxide sealers, they exert an antimicrobial effect by releasing hydroxyl ions and increasing the pH. **Objective:** The objective of this study was to evaluate and compare the antimicrobial activity of a calcium hydroxide-based sealer and two bioceramic sealers against *Porphyromonas gingivalis*, *Enterococcus faecalis*, and *Candida albicans* biofilms. **Materials and Methods:** The sealers were dissolved in sterile saline to obtain supernatants. Biofilm formation assays, colony counting, and real-time polymerase chain reaction (PCR) were performed to evaluate the antimicrobial activity of each supernatant. The data were analyzed using one-way analysis of variance. **Results:** All sealers exerted effects against all three microbial biofilms. The biofilm formation assays showed that the bioceramic sealers were more effective against *P. gingivalis* and *E. faecalis* biofilms. In contrast, colony counting and real-time PCR showed that the calcium hydroxide sealer was significantly more effective than the bioceramic sealers. All tests showed that the calcium hydroxide sealer was more effective against *C. albicans*, with the colony count and real-time PCR results showing statistically significant differences. **Conclusion:** The calcium hydroxide-based sealer was more effective than the bioceramic sealers in eradicating pathogenic root canal biofilms.

**Keywords:** *Antimicrobial activity, bioceramic sealer, calcium hydroxide-based root canal sealer, Candida albicans, Enterococcus faecalis, Porphyromonas gingivalis*

## Introduction

Microorganisms and microbial products are the main etiologic factors associated with pulp disease and periapical lesions.[1] Gram-negative anaerobic bacterial species, one of which is *Porphyromonas gingivalis*, are often found in primary infections with necrotic pulp.[2-4] In secondary infections or apical periodontitis lesions in teeth that have undergone endodontic treatment, *Enterococcus faecalis* is the most frequently detected bacterium,[5-7] while *Candida albicans* is the most common fungal species.[3,6]

Bacterial infections in the root canal may cause periapical and pulp inflammation and lead to failure of a previous root canal treatment.[8] Even well-performed endodontic treatments may fail to completely eradicate persistent bacteria that cannot be reached by instruments or are resistant to disinfection procedures.[6] Microbes in persistent infection cases, such as *E. faecalis* and *C. albicans*, can invade and colonize dentin, live in conditions of nutrient deficiency, and resist calcium hydroxide treatments.[9-11]

Root canal treatments are performed to eliminate biofilms, eradicate infections, and prevent microorganisms from infecting or reinfecting root canals and periradicular tissue[5,12] by filling and sealing the root canal spaces.[13] However, complex root canal anatomical variations, such as isthmuses and canal ramifications, are often undetected, making the complete elimination of root canal bacteria uncertain.[14,15] Therefore, root canal filling materials should have the ability to eradicate biofilms and residual bacteria after instrumentation and root canal irrigation.[16-18]



**How to cite this article:** Suwartini T, Santoso J, Widyarman AS, Ratnasari D. Efficacy of bioceramic and calcium hydroxide-based root canal sealers against pathogenic endodontic biofilms: An *in vitro* study. Contemp Clin Dent 2021;XX:XX-XX.

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**Accepted :** 19-Jul-2021
**Published :** ***

***Address for correspondence:***
*Dr. Jessica Santoso, Departemen Konservasi Gigi, Fakultas Kedokteran Gigi, Universitas Trisakti, Kampus B – Jl. Kyai Tapa 260, Grogol, Jakarta Barat 11440, Jakarta, Java, Indonesia.*
*E-mail: drg.jessica.santoso@gmail.com*

AQ5

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Root canal sealers are used in conjunction with biologically acceptable solid or semisolid obturating materials to achieve adequate sealing of the root canal system.[19] Sealers with excellent sealing ability and antibacterial activity are required to control endodontic infections, inhibit harboring residual bacterial growth, prevent nutrient leakage and root canal reinfection, and facilitate the healing process of apical and periapical tissues.[8,16,17]

Calcium hydroxide-based sealers have antimicrobial properties[20,21] and osteogenic-cementogenic potential.[20,22] Calcium hydroxide exerts an antibacterial effect by releasing hydroxyl ions and increasing pH levels.[23,24] Previous studies have shown that calcium hydroxide root canal sealers have a wide range of antibacterial effects and lower cytotoxicity than other sealers. Their disadvantage, however, is that they dissolve more easily, forming gaps inside the root canal,[25] and thus do not meet Grossman's criteria for an ideal root canal sealer.[21]

In recent years, bioceramic materials have been developed as root canal sealers. These materials are calcium silicate-based cement with the addition of several oxide components.[20] They are known to have bioactive properties that can stimulate tissue repair and induce mineralization and are therefore considered suitable for root canal sealing applications.[8,26]

Bioceramic sealers are also advantageous because they are biocompatible, bioactive, nontoxic, presented an alkaline pH, and dimensionally stable with minimal expansion.[27,28] The two main features of these materials are their hydraulic nature and their reactivity due to the formation of calcium hydroxide that is leached in a solution.[26] Their hydrophilic properties mean that they are not sensitive to moisture and blood contamination, which makes them ideal for the treatment of root canals and tubules, which are naturally moist.[29] After setting, they become hard and insoluble, providing excellent long-term sealing.[30] Moreover, they provide pH values above 12 due to a hydration reaction whereby calcium hydroxide is formed and breaks down into calcium and hydroxyl ions.[30]

Although several *in vitro* studies have reported varying degrees of antimicrobial activity of bioceramic sealers, safe conclusions cannot be drawn because of the high heterogeneity that characterize these studies.[13] Like calcium hydroxide sealers, bioceramic sealers exert an antimicrobial effect by releasing hydroxyl ions and increasing the pH.[31] However, only a few studies have investigated the effects of bioceramic sealers against *P. gingivalis*, *E. faecalis*, and *C. albicans*. Therefore, this study aimed to examine the differences in the ability of two bioceramic sealers and a calcium hydroxide-based sealer to eradicate *P. gingivalis*, *E. faecalis*, and *C. albicans* biofilms.

## Materials and Methods

### Sample preparation and study design

A laboratory experimental study with a posttest-only control design was conducted to investigate the efficacy of root canal sealers against endodontic biofilms. The root canal sealers tested were BioRoot™ RCS (Septodont, France), Sure-Seal Root™ (Sure Dent, South Korea), and Sealapex™ (Kerr, USA). Table 1 shows the chemical composition and characteristics of the sealers. Each sealer was prepared according to its manufacturer's instructions, distributed to three silicone molds with a diameter of 7 mm and a depth of 3 mm, and incubated at 37°C under humid conditions for 24 h. After setting, the sealer blocks were powdered using a mortar and pestle and then dissolved in a sterile saline solution (Otsu NS NaCl 0.9%; Otsuka, Indonesia) to obtain suspensions in concentrations of 50 mg/mL. Each suspension was homogenized for 10 min and then centrifuged at 4000×g at 25°C for 10 min to obtain a supernatant. The supernatants were then filtered with 0.22-µm filters (Minisart® single filter; Sartorius, Germany) to remove any deposits.

### Pathogen cultures

Quantities of 50 µL of *P. gingivalis* (ATCC® 33277™) and *E. faecalis* (ATCC® 29212™) bacterial suspensions were cultivated aerobically in 1.9 mL of brain–heart infusion (BHI) broth (Sigma-Aldrich, USA). A total of 50 µL of *C. albicans* (ATCC® 10231™) suspension was cultivated in 1.9 mL of Sabouraud dextrose broth (Sigma-Aldrich, USA). All suspensions were homogenized using a vortex mixer (MX-S; DLAB Scientific, PRC) and then incubated at 37°C for 24 h. The cultures were diluted to an equivalent of optical density $(OD)_{600}$ 0.132 (McFarland 0.5 or $1.5 \times 10^8$ CFU/mL) in accordance with the inoculum density standards of the Clinical and Laboratory Standards Institute.[32]

### Biofilm formation assay

Quantities of 200 µL of suspensions were inoculated in 96-well microplates (Biologix, USA) and incubated again under anaerobic conditions at 37°C for 24 h to form biofilms. The supernatants of bacteria and fungi that had been incubated were discarded until only the biofilms at the bottoms of the well plates remained. Subsequently, the supernatants of the three sealers were distributed 200 µL per well, repeated six times for each experimental group, and then incubated at 37°C for 24 h.

After incubation for 24 h, four out of six wells containing biofilms and sealer supernatants of each experimental group were rinsed with 200 µL of phosphate-buffered saline (PBS; VWR Life Science, USA). Suspensions from the remaining wells were transferred into microtubes for colony counts and real-time quantitative polymerase chain reaction (qPCR). Biofilm staining was performed

**Table 1: Compositions, manufacturers, and lot numbers of the tested sealers**

| Material | Composition | Producer | Lot number | Notes |
|---|---|---|---|---|
| BioRoot™ RCS | Powder: Tricalcium silicate, zirconium dioxide, and povidone<br>Liquid: Water, calcium chloride, and polycarboxylate | Septodont, France | B23103 | Bioceramic sealer |
| Sure-Seal Root™ | Calcium silicate, calcium aluminate, calcium aluminoferrite, calcium sulfate, radiopacifier, and thickening agent | Sure Dent, South Korea | WR953100 | Bioceramic sealer |
| Sealapex™ | Base paste: N-ethyl-o-toluene sulfonamide, calcium oxide, zinc oxide, and zinc distearate<br>Catalyst paste: Methyl salicylate, 2,2-dimethylpropane-1,3-diol, and isobutyl salicylate | Kerr, United States | 7081108 | Calcium hydroxide-based sealer |

RCS: Root canal sealer

with 200 µL of 0.5% crystal violet solution (Merck, USA) in each well for 15 min and then rinsed again with PBS. A total of 200 µL of absolute ethanol (EMSURE®; Merck, USA) was inserted into each well, and absorption measurements were conducted using a microplate reader (MP96; Safas, Monaco) at a wavelength of 595 nm.

### Counting of microbial colony-forming units

Aliquots of 100 µL of each treatment were pipetted to perform two serial 100-fold dilutions. A total of 2 µL of the diluted suspension was plated on a sterile BHI agar medium (Oxoid, USA). The suspensions in all Petri dishes (Iwaki Glass, Indonesia) were incubated at 37°C for 24 h (anaerobically for the *P. gingivalis* and *E. faecalis* suspensions). The number of bacterial and fungal colonies formed was observed, calculated, and converted to colony-forming units per milliliter.

### Real-time quantitative polymerase chain reaction

Bacterial and *Candida* DNA extraction was performed using the heat-shock method. The suspensions were centrifuged at 4500×g for 15 min. The supernatants formed were then discarded to get a pellet filled with pathogens. The pellets were resuspended with 100 µL of $ddH_2O$ and then homogenized for 5 min. Microtubes were heated in a dry block thermostat (Bio TDB-100; Biosan, Latvia) at 100°C for 20 min and then immediately placed in an ice bath for 10 min. After the extraction, the samples were homogenized again with a vortex mixer. Centrifugation was performed again at 10,000×g for 2 min. The supernatants containing DNA were transferred into new microtubes and stored at 4°C. The samples were evaluated after 24 h.

Mixtures of 20 µL were prepared for the qPCR test, each containing 2 µL of DNA, 10 µL of qPCR Mix (HOT FIREPol® SolisGreen qPCR Mix; Solis BioDyne, Estonia), 6 µL of nuclease-free water, 1 µL of forward primer, and 1 µL of reverse primer. The primers used were AGGCAGCTTGCCATACTGCG (forward) and ACTGTTAGCAACTACCGATGT (reverse) for *P. gingivalis* with an amplicon length of 127 bp, 5'-GTT TAT GCC GCA TGG CATAAG AG-3' (forward) and 5'-CCG TCA GGG GAC GTT CAG-3' (reverse) for *E. faecalis* with an amplicon length of 310 bp, and CCC AGT CTT TCA CAA GCA GTA AAT (forward) and GTA AAT GAG TCA TCA ACA GAA GCC (reverse) for *C. albicans* with an amplicon length of 356 bp.

The mixtures were homogenized and distributed to 48-well PCR plates (Biologix, USA). *P. gingivalis, E. faecalis*, and *C. albicans* were identified by PCR amplification of the 16S rRNA gene. Real-time PCR was performed using a thermal cycler (Applied Biosystems, StepOne Real-Time PCR System™; Thermo Fisher Scientific, USA) with SYBR® Green I fluorophore. The program, temperature, and plate design were set on a computer connected to the thermocycler. In each well, the gene expression intensity was measured and the threshold cycle (Ct) values, that is, the relative values representing the number of cycles in which the amplified DNA reaches a threshold level, were obtained. The Ct values were then converted to colony-forming units per milliliter using the standard curve of each microbe.

### Statistical analysis

The data obtained from the biofilm formation assays, colony counts, and real-time PCR, all ratio scale data, were tested for normality using the Shapiro–Wilk test. One-way ANOVA test was performed, followed by Tukey's honestly significant difference *post hoc* test were used to determine the significance of the differences between experimental groups. The level of statistical significance was set to $P < 0.05$. The statistical analysis was performed using IBM SPSS® Statistics version 25 (IBM, USA). AQ7 AQ8

## Results

### *Porphyromonas gingivalis* biofilms

The results of the biofilm formation assays showed that the BioRoot RCS bioceramic sealer was the most effective in eradicating *P. gingivalis* biofilms (OD: 0.155), followed by the Sure-Seal Root bioceramic sealer and the Sealapex calcium hydroxide-based sealer. However, the colony count results [Figure 1] showed that Sealapex was the most effective against *P. gingivalis* ($7.5 \times 10^6$ CFU/mL), followed by BioRoot RCS and Sure-Seal Root. The difference was statistically significant ($P < 0.05$). Real-time PCR also showed that Sealapex was significantly more

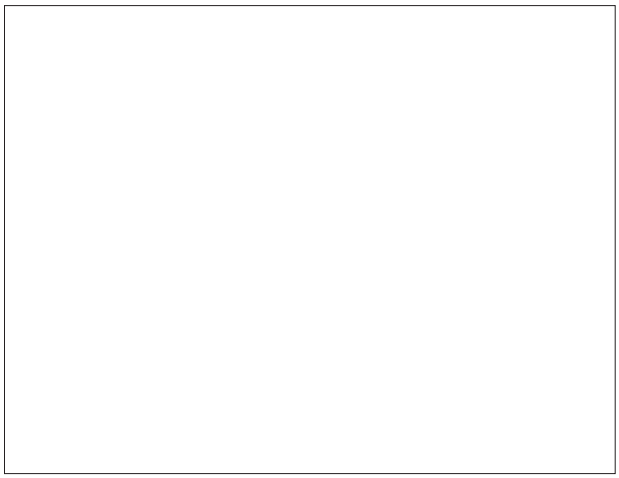

**Figure 1:** Colony counts of *Porphyromonas gingivalis* on brain–heart infusion agar plates with treatments in duplicate

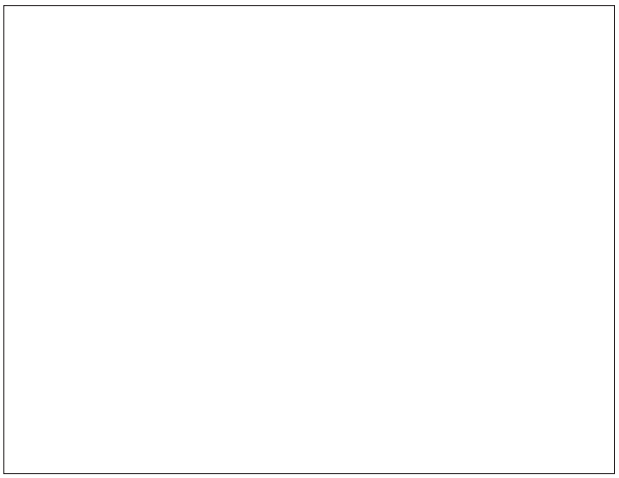

**Figure 2:** Antimicrobial activity measurement results of the root canal sealers against *Porphyromonas gingivalis*. (a) Biofilm formation assay; (b) Colony counts; (c) Real-time polymerase chain reaction. The error bars indicate standard deviations of the means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

effective ($2.345 \times 10^4$ CFU/mL) than both bioceramic sealers ($P < 0.01$). Figure 2 shows the results of the activity of the three root canal sealers against *P. gingivalis* biofilms and the statistically significant differences between the groups.

### *Enterococcus faecalis* biofilms

The biofilm formation assays showed that Sure-Seal Root was the most effective in eradicating *E. faecalis* biofilms (OD: 0.181), followed by BioRoot RCS and Sealapex. The antibacterial effect of both bioceramic sealers was significantly stronger than that of Sealapex ($P < 0.01$). However, qPCR showed that Sealapex was the most effective against *E. faecalis* ($1.38 \times 10^5$ CFU/mL), followed by Sure-Seal Root and BioRoot RCS. Moreover, the colony count results showed that Sealapex was highly effective against *E. faecalis*, with 0 CFU/mL formed [Figure 3]. In both tests, the antibacterial effect of Sealapex was significantly stronger than that of BioRoot RCS ($P < 0.05$). Although Sealapex has better antibacterial effect, it was not statistically significant when compared to Sure-Seal Root. The results of the antimicrobial activity measurements of three root canal sealers against *E. faecalis* biofilms and the statistically significant differences between the groups are shown in Figure 4.

### *Candida albicans* biofilms

The biofilm formation assays showed that Sealapex was the most effective in eradicating *C. albicans* biofilms (OD: 0.45), followed by BioRoot RCS and Sure-Seal Root. However, the differences between the sealers were not statistically significant. The colony count [Figure 5] and qPCR results also showed that Sealapex was the most effective (0 CFU/mL and 496.172 CFU/mL, respectively). In both tests, the antimicrobial effect of Sealapex was significantly stronger than that of Sure-Seal Root ($P < 0.05$). Sealapex also performed better compared to BioRoot RCS, although it was not statistically significant. Figure 6 shows the results of the antimicrobial activity measurements of the three root canal sealers against *C. albicans* biofilms and the statistically significant differences between the groups.

## Discussion

Both bioceramic and calcium hydroxide-based sealers are able to inhibit bacterial growth at the concentration of 50 mg/mL in concordance with prior studies.[8,17,31] The pH measurement done for each sealer supernatant showed a value of 11.55 for the BioRoot RCS suspension, 11.64 for Sure-Seal Root, and 12.47 for Sealapex. An alkaline pH causes denaturation of cytoplasmic membrane proteins, lipid peroxidation, and inhibition of DNA replication and acts as a physical barrier that restricts microbial growth.[33]

The biofilm formation assays showed that the bioceramic BioRoot RCS sealer was the most effective against *P. gingivalis*, followed by the bioceramic Sure-Seal Root sealer and the calcium hydroxide-based Sealapex sealer. These findings are comparable with the results of a previous study using biofilm assays that reported that calcium silicate-based sealers have a strong antimicrobial effect against Gram-positive *E. faecalis* along with Gram-negative *P. gingivalis* and *Porphyromonas endodontalis* bacteria.[5]

Antibacterial activity of BioRoot RCS against *P. gingivalis* is due to its tricalcium silicate, povidone, and zirconium oxide contents. When in contact with a liquid, tricalcium silicate reacts and produces hydroxyl ions, which increase the pH in the root canal system[26] and eradicate Gram-negative bacteria such as *P. gingivalis* by damaging their cell membranes, inhibiting their lipopolysaccharides, and denaturing their proteins.[23,24] Povidone does not have microbicidal properties but slows the release of excess ions, thus maintaining long-term antimicrobial activity.[34] Zirconium oxide damages bacterial membranes and prevents further growth.[35]

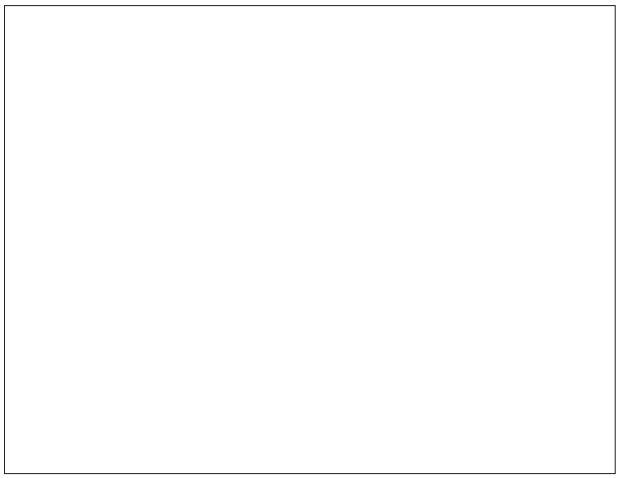

**Figure 3: Colony counts of *Enterococcus faecalis* on brain–heart infusion agar plates with treatments in duplicate**

**Figure 5: Colony counts of *Candida albicans* on brain–heart infusion agar plates with treatments in duplicate**

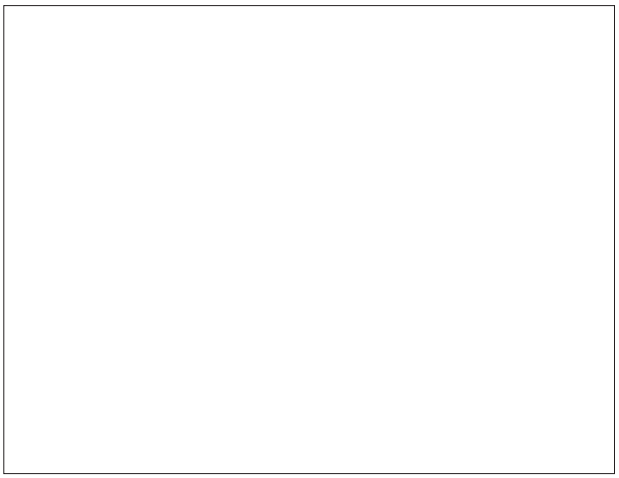

**Figure 4: Antimicrobial activity measurement results of the root canal sealers against *Enterococcus faecalis*. (a) Biofilm formation assay; (b) Colony counts; (c) Real-time polymerase chain reaction. The error bars indicate standard deviations of the means. **P* < 0.05; ***P* < 0.01; ****P* < 0.001**

**Figure 6: Antimicrobial activity measurement results of the root canal sealers against *Candida albicans*. (a) Biofilm formation assay; (b) Colony counts; (c) Real-time polymerase chain reaction. The error bars indicate standard deviations of the means. **P* < 0.05; ***P* < 0.01; ****P* < 0.001**

The results of colony count and real-time PCR (qPCR) showed that Sealapex was the most effective against *P. gingivalis*, followed by the two bioceramic sealers. Previous studies revealed that the antibacterial activity of calcium hydroxide depends on the total concentration and release rate of hydroxyl ions.[36] Calcium hydroxide-based sealers can break down into calcium ions and hydroxyl ions, causing an increase in pH up to 12.5, which can damage the microbial cytoplasmic membrane.[37] This high pH is obtained within 1 h and can last for 30 days.[21] Hydroxyl ions are highly oxidant free radicals that are highly reactive to cytoplasmic membrane biomolecules, thus compromising the integrity of the cytoplasmic membrane of bacteria and inhibiting the lipopolysaccharides of Gram-negative bacteria.[38] Moreover, when reacting with carbon dioxide, calcium ions can block the source of respiration of anaerobic bacteria.[36]

Sure-Seal Root was the most effective against *E. faecalis* bacteria, followed by BioRoot RCS and Sealapex in the biofilm formation assays. A previous study comparing traditional and bioceramic sealers found that calcium silicate-based sealer (Endoseal MTA; Maruchi, South Korea) was more effective against *E. faecalis* than other sealers, including calcium hydroxide-based sealers, due to the oxidizing components of calcium silicate-based sealers, which exert strong activity against both Gram-positive and Gram-negative bacteria.[8] The effect of Sure-Seal Root against *E. faecalis* is due to its calcium silicate, calcium aluminate, and calcium sulfate contents. Calcium silicate is effective against bacteria that are tolerant of alkaline conditions.[39] The oxide components of bioceramic sealers damage the cell walls of Gram-positive bacteria and increase the permeability of molecules into their cytoplasm.[40] These components also facilitate the penetration of calcium hydroxide into the cytosol and the denaturation of bacterial DNA and proteins.[24]

Colony counting and qPCR showed that the calcium hydroxide-based Sealapex was the most effective against

*E. faecalis*, followed by Sure-Seal Root and BioRoot RCS. These results are in line with previous studies which found that calcium hydroxide-based sealers showed excellent antimicrobial activity after 24, 48, and 72 h and 7 days using agar diffusion tests.[41,42] Similar results were obtained using direct contact tests, which showed that Sealapex acted against *E. faecalis* within 24 h[43] and was still more effective than other sealers after 7 days.[27] This is probably due to the release of hydroxyl ions, which create an unfavorable environment for the growth of microorganisms by increasing the pH.[41,42] The antimicrobial mechanism of calcium hydroxide-based sealers is influenced by the speed at which they break down into calcium ions and hydroxyl ions.[44] The decomposition of hydroxyl ions results in a high pH environment, thus inhibiting enzymatic activity, which is important for the metabolism and growth of microbes, as well as cell division.[21]

The biofilm assays, colony formation counts, and qPCR showed that Sealapex was the most effective against *C. albicans*, followed by BioRoot RCS and Sure-Seal Root. These results are consistent with those of a previous study reporting that calcium hydroxide had stronger effects against *E. faecalis* and *C. albicans* than MTA and Portland cement.[45] Its activity against *C. albicans* is due to the formation of inhibition zones.[46,47] Sulfonamides contained in Sealapex may increase its antibacterial and antifungal activity. Sulfa antibacterial agents can inhibit the formation and growth of *C. albicans* biofilms, which are usually more resistant to antifungal agents than planktonic cells,[48] by preventing the biosynthesis of folic acid.[49] As eukaryotic microbes such as fungi synthesize folate *de novo*, inhibition of folate biosynthesis causes folate deficiency, inhibiting cell growth.[50] Sulfa drugs block the folate pathway to dihydropteroate synthase enzyme, which *C. albicans* needs to convert para-aminobenzoic acid to dihydrofolate. The interruption of the folate pathway in *C. albicans* can also inhibit the biosynthesis of ergosterol.[48] Without ergosterol, which maintains the integrity of the cell membrane, its permeability increases.[51]

Previous studies had investigated the antibacterial effectiveness of calcium silicate-based root canal sealers with varied results, mostly against *E. faecalis*.[1,52] Our study examined the antimicrobial activities of both bioceramic and calcium hydroxide-based root canal sealers against *E. faecalis*, and lesser studied root canal pathogens such as *P. gingivalis* and *C. albicans*, which are mostly found in primary and persistent infection, respectively.[53,54] The three testing methods performed in this study were biofilm formation assays, counting of colonies formed on BHI agar media, and real-time PCR. Similarity of the results obtained by different methods would determine the quality and validity of the conclusion.

However, a shortcoming in our study is that the biofilm formation assays produced contradictory results with the colony counts and qPCR regarding the sealers' effectiveness against *P. gingivalis* and *E. faecalis*. This discrepancy may have been caused by deposits produced by the sealer supernatants, which implies that filtering with 0.22-μm filters did not ensure the supernatants were free from deposits. In previous studies, most deposits found in Sealapex and BioRoot RCS were due to calcium precipitation.[55,56] As biofilm formation assay measures biomass by absorption of crystal violet stains,[57] this may lead to readings of both precipitation and biofilm mass formed at the bottom of the well plate, causing a higher OD. This finding, however, was not seen in the *C. albicans* experimental groups, although it should be noted that in the biofilm formation assay, there were no significant differences between the three tested sealers. Future studies should take care to obtain deposit-free supernatants, therefore increasing the accuracy of biofilm formation assay results.

Molecular methods for microbial identification, such as qPCR, have advantages over culture methods. Real-time PCR can identify microbes more accurately, does not require microbial cultures, and thus can detect both cultivable and noncultivable species, and can be performed quickly on many samples.[58] Culture methods, on the other hand, can only detect microbes that can be cultivated and form colonies.[57,58] Differences in the detection methods may sometimes cause discrepancies between the results of qPCR and those of cultivation methods.[57] Previous studies indicate that such discrepancies in both methods may be explained by the inability of cultivation methods to distinguish between close related bacteria, the different threshold levels of both methods, and the problems of keeping pathogenic bacteria viable, which is required for standard cultivation.[59] In this study, however, colony counting and qPCR produced mostly consistent results. In this study, however, the results of colony counting and qPCR are mostly consistent. The possible explanations to these findings are the use of standard reference strain (ATCC) instead of microbial isolates and that the pathogens used in this study are facultative anaerobes, which are easier to cultivate and kept viable.

Within the limitations of this study, most tests showed that Sealapex was the most effective against all pathogens, namely *P. gingivalis*, *E. faecalis*, and *C. albicans*. However, as antimicrobial properties are only one of the many properties required for an ideal root canal sealer, other properties of bioceramic sealers, such as dimensionally stable and insoluble in tissue fluids,[16] could make this type of sealer worth considering as a root canal obturation materials.[20,60] Therefore, further *ex vivo* studies should be done to examine and compare the antimicrobial effect of the root canal sealers on extracted human teeth which will be shaped, cleaned, and obturated using the tested sealers, as environment inside the root canal along with other factors and variables may affect the final result differently. Further

studies should also investigate the increase or decrease in the antimicrobial effects of each sealer over time, as both bioceramic and calcium hydroxide-based root canal sealers' antimicrobial effects were based on the release of hydroxyl ions and increase pH levels that were obtained over time. Moreover, similar studies could examine the sealers' effects against other root canal pathogens.

## Conclusion

All three root canal sealers had antimicrobial effects. Real-time PCR showed that the calcium hydroxide-based sealer was more effective than the bioceramic sealers against *P. gingivalis* biofilms. Both colony counts and qPCR showed that the calcium hydroxide-based sealer was also more effective against *E. faecalis*. Furthermore, all three tests performed showed that it was also the most effective against *C. albicans* biofilms. These results suggest that calcium hydroxide-based sealer was the most effective against all pathogenic root canal biofilms studied.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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