

Original Article



Combined Effect of Calcium Hydroxide with Chitosan, Pantoprazole and Nisin Against *Enterococcus Faecalis* Biofilm

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Abstract

Introduction: Endodontic treatment in the presence of resistant *Enterococcus faecalis* biofilms remains challenging due to the organism's ability to form resilient biofilms, leading to persistent infections and treatment failure. Calcium hydroxide (CH) is widely used as an intracanal medicament, but biofilm formation may reduce its effectiveness. Combining CH with antibacterial agents such as chitosan, pantoprazole, and nisin may improve antimicrobial efficacy. This study evaluated the combined effects of CH with these agents on *E. faecalis* biofilm eradication.

Methods: *E. faecalis* (ATCC 29212) was cultured in heart brain infusion broth at 37 °C. CH, nisin, pantoprazole, and chitosan were prepared, and minimum inhibitory concentrations (MICs) were determined by broth microdilution. Biofilm formation was quantified using crystal violet staining, and changes in biofilm-related gene expression (*gelE* and *efaA*) were assessed by quantitative PCR. Statistical analyses were performed using STATA and ANOVA.

Results: Nisin exhibited the strongest growth inhibition (MIC: 125 µg/mL). Chitosan caused the greatest reduction in biofilm biomass (mean OD: 0.262 ± 0.078, **P* < 0.001). The combination of chitosan with CH and EDTA reduced OD to 0.235 ± 0.065, significantly more than other combinations (**P* < 0.001). Chitosan and nisin significantly downregulated *gelE* and *efaA* expression (**P* < 0.05).

Conclusion: The combination of CH with chitosan significantly reduced *E. faecalis* biofilm biomass and suppressed biofilm-related gene expression, suggesting its potential as an adjunctive agent in endodontic therapy. Nisin also demonstrated significant antimicrobial activity, while pantoprazole had relatively limited effects. These findings support the inclusion of chitosan in intracanal medicament strategies to improve treatment outcomes.

Keywords: Endodontic infection, Antibiofilm activity, Biofilm-associated genes, Hydroxyl ions, Persistent apical periodontitis

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Introduction

Apical periodontitis is a common complication of dental pulp infection, usually caused by microbial invasion of the root canal system.¹ The main goal of root canal therapy is to eliminate microorganisms and prevent reinfection. However, in some cases, infection persists even after treatment, often due to the presence of microbial biofilms within the canal system.²⁻³

Biofilms are aggregates of microbes encased in a self-produced extracellular matrix, which renders them highly resistant to antimicrobial agents and mechanical cleaning techniques. This characteristic noticeably decrease the effectiveness of standard treatments and contributes to the

development of chronic and resistant infections.⁴ Within biofilms, microorganisms exhibit greater resistance compared to their planktonic (free-floating) state, making complete disinfection of the root canal system a serious challenge.⁵⁻⁶

Calcium hydroxide (CH) is one of the most commonly used intracanal medicaments in root canal therapy.⁷ Its antimicrobial action is attributed to the creation of an alkaline environment and the release of hydroxyl ions, which damage bacterial membranes, denature proteins, and harm DNA.⁸⁻⁹ However, evidence suggests that free calcium ions (Ca²⁺) may increase bacterial adhesion and thereby enhance biofilm stability.¹⁰⁻¹¹



This limitation clearly indicates the need for complementary approaches to enhance the efficacy of CH against biofilms.¹²⁻¹³ Compounds such as chitosan, pantoprazole, and nisin have been considered as potential enhancers of the antimicrobial effects of CH.¹⁴⁻¹⁶ Chitosan, a natural polysaccharide with intrinsic antimicrobial and antibiofilm properties, can also act as an effective drug carrier.¹⁷⁻¹⁸ Pantoprazole, a proton pump inhibitor, may enhance CH activity, possibly by suppressing bacterial defense mechanisms.¹⁹ Nisin, a lantibiotic peptide, exerts potent bactericidal activity—especially against Gram-positive organisms—by forming pores in the bacterial membrane and inhibiting cell wall synthesis.²⁰

The root canal microbiota is part of a diverse oral microbiome that includes polymicrobial communities capable of forming resistant biofilms and causing persistent infections. Oral bacteria contribute to the development of apical periodontitis by inducing host inflammatory responses and destroying periapical tissue. Furthermore, these biofilms are resistant to both antimicrobial agents and the host immune defense system. In particular, *Enterococcus faecalis*, a Gram-positive facultative anaerobe, plays a significant role in refractory apical periodontitis due to its adaptability, strong adhesion, ability to survive under harsh conditions, and resistance to common disinfectants. Overall, the root canal microbiota and its constituent microbial communities are capable of forming stable biofilms that lead to persistent infections.²¹

Among these microorganisms, *E. faecalis* is commonly isolated from cases of failed root canal treatment.²² The remarkable adaptability of this organism allows it to survive in harsh conditions such as high pH, nutrient deficiency, and oxidative stress. Furthermore, its ability to penetrate dentinal tubules, strongly adhere to canal surfaces, and form resistant biofilms increases its resistance to conventional disinfectants and facilitates colony regrowth—characteristics that also make it a major factor in refractory apical periodontitis.²³⁻²⁴

Due to its clinical significance, *E. faecalis* is regarded as an appropriate model for testing new antimicrobial agents aimed at eradicating biofilms.²⁵⁻²⁶ Previous research has investigated the antimicrobial properties of chitosan, pantoprazole, and nisin against *E. faecalis* individually. Nevertheless, there has been insufficient systematic investigation into the combined effects of these agents with calcium hydroxide on biofilm eradication, alongside simultaneous assessment of biofilm-related gene expression. This study aimed to evaluate the combined efficacy of calcium hydroxide with chitosan, pantoprazole, and nisin in eliminating *E. faecalis* biofilms. Also, we identified more effective strategies for managing resistant infections following root canal therapy.

Materials and Methods

Study Design and Ethical Approval

This experimental study was carried out at Kurdistan University of Medical Sciences and received ethical approval from the Institutional Review Board (IRB).

Bacterial Strain and Cultivation Conditions

Enterococcus faecalis (ATCC 29212) served as the bacterial strain for all experimental procedures. Initially, a pre-culture was established by inoculating a single colony into brain heart infusion (BHI) broth, followed by overnight incubation at 37 °C under aerobic conditions with shaking at 150 rpm. After this growth period, the culture was adjusted to a concentration of 10⁶ CFU/mL by assessing the optical density at 600 nm using spectrophotometry. To ensure accuracy in bacterial concentration, direct colony counts were performed by plating on BHI agar, confirming the CFU/mL measurement. This precisely prepared bacterial suspension was used in all subsequent tests.

Preparation of Solutions and Drugs

Calcium hydroxide solution: To prepare a saturated calcium hydroxide solution, calcium hydroxide powder at a concentration of 1.6 g/L (equivalent to 21.6 mM) was dissolved in deionized water. The mixture was incubated at room temperature for 24 hours, followed by centrifugation to eliminate undissolved particles. The clear supernatant obtained was then used for biofilm assays.

Hydroxide ion (OH⁻) solution: To remove calcium ions from the saturated CH solution, ethylenediaminetetraacetic acid (EDTA) was added, and the mixture was incubated at room temperature for 24 hours. The presence of OH⁻ ions was verified using the Eriochrome Black T indicator.²⁷ The pH of all solutions was measured and documented to maintain stability and account for potential pH-dependent variations in antimicrobial activity, especially for pH-sensitive agents such as nisin.

Calcium ion (Ca²⁺) solution: This was prepared by neutralizing the CH solution with acetic acid, yielding a solution containing only Ca²⁺ ions at a final pH of 7.0. This pH was closely monitored to differentiate the effects of calcium ions from any antibacterial effects associated with pH changes.

Nisin, pantoprazole, and chitosan stock solutions were prepared under sterile conditions at concentrations of 10 mg/mL each for nisin and pantoprazole, and 5 mg/mL in 1% acetic acid for chitosan. These solutions were stored at appropriate temperatures until required for biofilm removal assays. Special care was taken with nisin solutions to maintain their activity within the optimal pH range (acidic to neutral). Table 1 summarizes the final

Table 1. Final Concentrations and pH of Compounds Used in Biofilm Eradication Assays

Compound	Final Concentration	pH
Calcium hydroxide (CH)	Saturated solution (~21.6 mM)	~12.5
OH ⁻ solution (CH + EDTA)	As prepared	~12.5
Ca ²⁺ solution (neutralized CH)	As prepared	7.0
Nisin	125 µg/mL (MIC)	5.5 – 6.0
Pantoprazole	According to MIC	According to MIC
Chitosan	According to MIC	According to MIC

concentrations and pH values of all compounds used in the biofilm eradication tests.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) for each solution and drug was determined using the broth microdilution technique in 96-well plates with U-shaped bottoms. Serial two-fold dilutions of each substance were prepared in BHI broth to a total volume of 100 μ L per well. The highest concentration was placed in the first well of each plate. A bacterial suspension of 100 μ L at a concentration of 10^6 CFU/mL was then added to each well, and the plates were incubated at 37 °C for 24 hours. The MIC was identified as the lowest concentration at which visible bacterial growth was completely inhibited. After the incubation period, results were assessed visually.²⁸⁻²⁹ Throughout all MIC assays, pH levels were monitored to maintain consistency, especially when testing pH-sensitive compounds.

Biofilm Formation and Treatment

Biofilms were cultivated in 96-well flat-bottom microtiter plates. Each well received 100 μ L of bacterial suspension at a concentration of 10^6 CFU/mL. The plates were then incubated at 37 °C in a humidified environment for 72 hours under aerobic conditions to facilitate biofilm formation. Following incubation, the biofilms were gently washed three times with phosphate-buffered saline (PBS) to remove nonadherent cells. The experimental setup included various groups, with biofilms exposed to different solutions and drugs for 24 hours. The groups were as follows:

- Control group: *E. faecalis* in BHI broth.
- CH solution group: *E. faecalis* in BHI broth with added CH solution.
- OH⁻ solution group: *E. faecalis* in BHI broth with OH⁻ solution.
- Ca²⁺ solution group: *E. faecalis* in BHI broth with added Ca²⁺ solution.
- Drug treatment group: *E. faecalis* in BHI broth with supplements of nisin, pantoprazole, or chitosan.

Post-treatment, biofilms were washed three times with PBS to eliminate nonadherent bacteria. Biofilm biomass was assessed using the crystal violet staining method. The biofilms were stained with 0.1% crystal violet solution for 15 minutes at room temperature. Excess stain was washed away with PBS, and the dye retained by the biofilm was solubilized in 95% ethanol. The optical density of the solubilized stain was measured at 590 nm using a microplate reader, with these values serving as a direct measure of biofilm biomass.³⁰ The pH of the supernatants was recorded before and after treatment to account for potential pH-dependent variations in biofilm removal.

Subinhibitory Concentration Assay

Subinhibitory concentrations (SICs) were established as levels just below the MIC, allowing for biofilm

development while maintaining cell viability. Cultures were exposed to sub-MIC concentrations for 72 hours, during which biofilm biomass was measured via crystal violet staining. Simultaneously, the expression of genes associated with biofilm formation was assessed using quantitative PCR (qPCR). Throughout all SIC treatments, pH was monitored to ensure accurate interpretation of antimicrobial effects.

Gene Expression Analysis by qPCR

To evaluate the impact of treatments on biofilm-associated genes, RNA was extracted from biofilm samples using the Qiagen RNeasy Kit. The RNA was then reverse-transcribed into complementary DNA (cDNA). Quantitative real-time PCR was performed using SYBR Green MasterMix according to the manufacturer's guidelines to quantify the expression levels of *gelE* and *efaA*. The primers used for each gene were as follows:

- *gelE*: Forward 5'-GGGGAACCGCTGAGGTTG-3', Reverse 5'-TCGAGACAGAGAGGAGAGAG-3'
 - *efaA*: Forward 5'-TGCTTGAGGCTTATCCAGG-3', Reverse 5'-CCTGAGGGAAGGAGAGAGAG-3'
- 16S rRNA (housekeeping gene): Forward 5'-CGGTTGAGTCAGGAGGTTT-3', Reverse 5'-AGGACGGGCCAGAGTTTAA-3'
- Expression levels of the target genes were normalized to 16S rRNA, and the $\Delta\Delta C_t$ method was used for calculations.²⁹⁻³⁰

Combination Treatment for Biofilm Eradication

To evaluate the efficacy of combination regimens, various mixtures including CH solution, OH⁻ solution, Ca²⁺ solution, and drug compounds were applied to mature biofilms for 24 hours. Biofilm biomass was measured using the crystal violet assay, and the most effective combinations were selected for further evaluation.^{31,32} These selected regimens were subsequently analyzed to investigate possible synergistic effects in eradicating *E. faecalis* biofilms. The pH of each combination was also measured to account for possible interactions between pH and antimicrobial efficacy.

Statistical Analyses

Data were analyzed using STATA software version 12. Results are presented as mean $\pm$ standard deviation for each treatment group. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons, with * $P < 0.05$ considered significant. All experiments were conducted in triplicate to ensure reliability and reproducibility. The analyses compared the effects of treatments on biofilm formation, antimicrobial efficacy, bacterial load, and gene expression.

Results and Discussion

In this study, the effects of pantoprazole, chitosan, and nisin, both alone and in combination with calcium hydroxide (CH) components, were evaluated on the elimination of *Enterococcus faecalis* biofilms. The results indicated that OH⁻ alone and CH combined with chitosan

were the most effective regimens for reducing biofilm biomass and inhibiting bacterial growth. The minimum inhibitory concentration (MIC) was determined using the broth microdilution method, and the results showed that nisin had the lowest MIC (125 µg/mL), indicating the strongest antimicrobial activity, followed by pantoprazole (250 µg/mL) and chitosan (800 µg/mL) (Figure 1B).

Nisin is a bacteriocidal peptide that causes leakage of cellular contents and cell death by creating pores in the cell membrane.^{33,34} The primary activity of this substance is against Gram-positive bacteria, with less pronounced effects on Gram-negative organisms.³⁵ However, it should be noted that the activity and stability of nisin are strongly influenced by environmental pH, and alkaline conditions lead to the degradation of this substance. Therefore, pH was carefully controlled during the experiments (Table 1). Optimal performance and stability are observed under acidic conditions.^{36,37} Biofilm removal, assessed by optical density (OD) measurements, showed that chitosan caused the greatest reduction in biofilm biomass (mean OD: 0.262 ± 0.078), which was significantly lower than that observed for nisin (0.327 ± 0.092) and pantoprazole (0.320 ± 0.108) (* $P < 0.001$, Figure 1A). These results suggest that while nisin exhibits strong direct antimicrobial activity, chitosan is more effective at disrupting the structure and stability of biofilms. However, it should be noted that the combination of these substances may be associated with limitations and side effects, including potential cytotoxicity, pH sensitivity (which was not measured in the experiment for nisin), or reduced activity under certain conditions. Therefore, caution should be

exercised when generalizing these findings to clinical applications. Among the materials examined in this study, the only agent that showed acceptable anti-biofilm activity was chitosan. In this regard, Wang et al, Zong et al and Ong et al also demonstrated in their studies that chitosan, through its cationic properties, creates electrostatic interactions with the negatively charged biofilm matrix, thereby disrupting bacterial adhesion and weakening structural integrity.³⁸⁻⁴⁰ Chitosan, through its amino and hydroxyl groups, can chelate metal ions, causing significant vulnerability and facilitating bacterial degradation. It also forms a strong bond with the bacterial cell wall, thereby increasing membrane permeability and ultimately leading to cell death.^{41,42} A direct relationship was observed between the reduction of *gelE* and *efaA* gene expression and the reduction of biofilm formation, indicating direct inhibition of biofilm development by chitosan and nisin. Since this study was conducted on single-species biofilms in vitro, future studies using in vivo and multispecies models are necessary to confirm the clinical relevance of the results. Moreover, the lack of a standard positive control in the experimental design limits the direct comparison of the efficacy of the tested compounds with established treatments. In this study, standard deviation values were not calculated for the determination of MIC or for gene expression data. Based on statistical analyses (Table 2), drug type, solution type, and incubation time were each significantly effective in reducing biofilm (* $P < 0.001$ for all three factors). In addition, significant interactions were recorded between drug and solution (* $P < 0.001$) as well as between

Table 2. Factors Affecting Biofilm Removal

Source	Sum of Squares	Df	Mean Square	F	P-Value
Drug	0.360	2	0.180	63.334	<0.001
Solution	1.199	2	0.600	210.941	<0.001
Time	0.173	1	0.173	61.040	<0.001
Solution Concentration	0.029	3	0.010	3.401	0.018
Drug-Solution Interaction	0.705	4	0.176	62.047	<0.001
Drug-Time Interaction	0.272	2	0.136	47.858	<0.001
Drug-Concentration Interaction	0.008	6	0.001	0.444	0.849
Solution-Time Interaction	0.002	2	0.001	0.414	0.661
Solution-Concentration Interaction	0.045	6	0.007	2.618	0.017

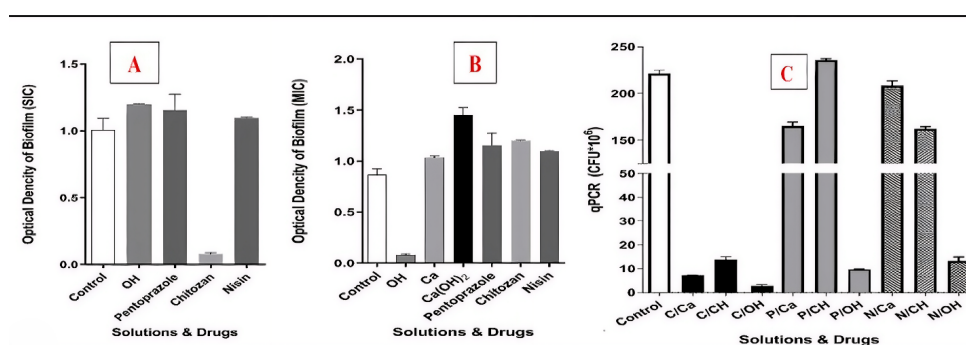


Figure 1. Effects of Treatments on *Enterococcus faecalis* Biofilms: (A) Optical Density Measurements at Subinhibitory Concentrations (SIC), (B) Optical Density Measurements at Minimum Inhibitory Concentrations (MIC) and (C) Biofilm-Associated Gene Expression Analysis by qPCR. Abbreviations: Ca, calcium; CH, calcium hydroxide; OH, hydroxide; P, pantoprazole; N, nisin; C, chitosan

solution and concentration ($*P^*=0.017$). These findings suggest that the combination of specific drugs with specific solutions effectively enhances biofilm removal. However, other interactions, such as drug concentration and solution time, were not statistically significant.

Regarding the different solution types, the hydroxide (OH^-) solution, obtained by chelating calcium ions from CH with EDTA, produced a significantly greater reduction in OD (0.235 ± 0.065) compared with CH alone (0.344 ± 0.093) and the calcium (Ca^{2+}) solution generated by reacting CH with acetic acid (0.356 ± 0.103) ($*P^* < 0.001$). No significant difference was observed between the CH and Ca^{2+} solutions ($*P^* = 1.000$). The significant and strong activity of the OH^- ion can be attributed to its effect in increasing the environmental pH, thereby creating conditions for protein denaturation, disruption of the cell wall, and inactivation of essential bacterial enzymes. This elevated pH also destroys the extracellular polymeric substance (EPS), a critical component of biofilm structural stability.⁴³ These observations align with those of Basrani et al, who attributed the antibacterial properties of CH to the release of OH^- and the associated rise in pH.⁴⁴ In contrast, although Ca^{2+} did not have a positive effect, it appeared to act as an ionic bridge within the biofilm matrix, strengthening the structure and enhancing biofilm integrity and connectivity. EPS, due to its negative charge, interacts with this positively charged divalent cation, leading to a significant increase in biofilm density.^{45,46} Mominejavid and colleagues reported that exposure to Ca^{2+} leads to denser and more granular biofilms with larger pores, resulting in an increase in total biomass and volume.⁴⁷

Despite the known roles of OH^- and Ca^{2+} in biofilm degradation and stabilization, the effects of CH as a standard intracanal medicament remain unclear, although results suggest limited efficacy against mature biofilms.^{47,48} In this regard, the study by Amini et al also showed that CH alone does not have a significant effect on reducing bacterial colony counts or biofilm biomass, although its activity increased significantly when combined with ciprofloxacin and betamethasone.⁴⁹ These results emphasize that to enhance the antimicrobial effect of $\text{Ca}(\text{OH})_2$ and its activity against mature biofilms, other antimicrobial agents should be added to this substance. Comparison of biofilm removal at 0 hours and 72 hours revealed a significant time-dependent decrease in OD ($*P^* < 0.001$). The combination of chitosan with the OH^- solution (CH + EDTA) demonstrated the highest efficacy in biofilm removal. By comparison, pantoprazole and nisin exhibited more limited antibacterial effects against mature *E. faecalis* biofilms. Pantoprazole, as a proton pump inhibitor (PPI), can enhance the antimicrobial activity of CH by disrupting pH homeostasis in *E. faecalis*.⁵⁰ The present findings are consistent with those of Divakar et al and Koparkar et al, who reported that combining CH with pantoprazole improves the inhibition of *E. faecalis* and may offer a promising intracanal medication strategy.^{51,52}

Gene expression analysis using qPCR revealed that

chitosan and nisin significantly downregulated the *gelE* and *efaA* genes, which are essential for biofilm formation and bacterial adhesion, compared to controls ($*P^* < 0.05$, Figure 1C). Pantoprazole and CH also reduced the expression of these genes, although the effects were less pronounced. The significant downregulation of *gelE* is particularly noteworthy, as this gene encodes gelatinase, an enzyme whose expression is tightly regulated by the *fsr* quorum sensing system (*fsrA* and *fsrB*) and is crucial for biofilm maturation and virulence in *E. faecalis*.^{53,54} Similarly, the reduction in *efaA* expression, which encodes an adhesin involved in biofilm formation,⁵⁵ further confirms the anti-biofilm mechanism of these compounds. The pronounced effect of nisin on gene expression may also be linked to its role as a signaling molecule in two-component regulatory systems, beyond its direct antimicrobial activity.^{56,57} These findings confirm that chitosan and nisin effectively inhibit biofilm-associated gene expression, highlighting their potential as adjunctive therapeutic agents.

Conclusion

In conclusion, this study demonstrates that combining CH with chitosan significantly enhances the eradication of *Enterococcus faecalis* biofilms compared to CH alone, with the most pronounced effect observed for the chitosan–CH combination—likely reflecting the synergistic action of chitosan's cationic chelating and membrane-disrupting properties alongside the biofilm-penetrating and extracellular matrix-degrading capacity of hydroxyl ions. This combination not only reduced biofilm biomass but also significantly downregulated the expression of the virulence genes *gelE* and *efaA*, which are critically involved in biofilm maturation and bacterial adhesion, thereby confirming its anti-biofilm mechanism at the molecular level. Nisin also demonstrated potent antimicrobial activity, as evidenced by its low minimum inhibitory concentration, yet its efficacy against mature biofilm disruption was inferior to that of chitosan, while pantoprazole exhibited only limited antibiofilm effects in this experimental setting. These findings underscore the importance of selecting appropriate adjunctive agents to overcome the intrinsic resistance of *E. faecalis* biofilms to conventional intracanal medicaments and highlight the clinical potential of incorporating chitosan into CH-based formulations to improve treatment outcomes in cases of persistent apical periodontitis. Nevertheless, given that this study was conducted in vitro using single-species biofilms, which do not fully replicate the complex polymicrobial environment of infected root canals, future research should prioritize validation in in vivo models and multispecies biofilm systems, alongside rigorous assessments of long-term stability, biocompatibility, and potential cytotoxicity, before clinical translation can be safely recommended. With further validation, the chitosan–CH combination holds promise as an effective adjunctive strategy for endodontic disinfection that may ultimately contribute to reducing the incidence of

persistent apical infections and improving the long-term success of root canal therapy.

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Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this study.

Ethical Approval

This study was approved by the Institutional Review Board of Kurdistan University of Medical Sciences (approval code: IRB-202) and conducted in accordance with institutional ethical standards.

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