# Antimicrobial efficacy of calcium hydroxide nanoparticle and nisin against *Enterococcus faecalis* in root canal therapy: an experimental study

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## ABSTRACT

**Introduction:** Failure in root canal therapy is often attributed to the incomplete elimination of pathogenic bacteria within infected canals. Enterococcus faecalis is the commonly identified bacteria in persistent cases. Its resistance to high pH environments is primarily mediated by a proton pump mechanism, which reduces the antimicrobial efficacy of calcium hydroxide ($Ca(OH)_2$). Nisin, an antimicrobial peptide, exerts its bactericidal action by disrupting bacterial plasma membranes, leading to cell lysis. The study aimed to evaluate antimicrobial efficacy of $Ca(OH)_2$ nanoparticles and their combination with nisin against E. faecalis. **Methods:** In the first phase, eight concentration of nisin (10-200 mg/mL) and three concentrations of $Ca(OH)_2$ nanoparticles (0.425-1.7 mg/mL) were tested using the agar diffusion method against E. faecalis on days 1 and 5. In the second phase, $Ca(OH)_2$ nanoparticles were combined with the most effective nisin concentration identified in phase one (100 and 150mg/mL), and antimicrobial activity was assessed on days 1, 3, 7, and 14. All experiments were conducted in triplicate to ensure reproducibility, and data were analyzed using one-way ANOVA followed by post hoc testing (α=0.05). **Results:** Nisin at 100 mg/mL and 150 mg/mL produced the largest inhibition zones (14.33 mm and 13.83 mm, respectively). The combination of $Ca(OH)_2$ nanoparticles and nisin demonstrated reduced antimicrobial activity compared with $Ca(OH)_2$ nanoparticles alone on days 7 and 14. **Conclusions:** Both $Ca(OH)_2$ nanoparticles and nisin exhibited antimicrobial effects against E. faecalis. Nisin alone was more effective than $Ca(OH)_2$ nanoparticles; however, their combination resulted in a diminished antimicrobial effect, suggesting a possible interaction or interference between the two agents.

## Keywords

Antimicrobial E. faecalis, calcium hydroxide, root canal medicament, nisin

# *Efektivitas antimikroba nanopartikel kalsium hidroksida dan nisin terhadap Enterococcus faecalis pada perawatan saluran akar: studi eksperimen*

## *ABSTRAK*

***Pendahuluan:*** *Kegagalan perawatan saluran akar umumnya disebabkan oleh pembersihan bakteri patogen yang tidak efektif di saluran akar yang terinfeksi. Bakteri E. faecalis adalah bakteri persisten yang menjadi etiologi pada kasus ini. Bakteri E. faecalis memiliki mekanisme pompa proton yang mampu melawan aksi ion $OH^-$, sehingga menghambat efektivitas $Ca(OH)_2$. Antimikroba lain, yaitu Nisin merupakan peptida yang mampu menembus membran plasma bakteri dan menyebabkan lisis sel. Tujuan penelitian ini adalah mengevaluasi efektivitas nanopartikel $Ca(OH)_2$ dan kombinasinya dengan nisin terhadap E. faecalis.* ***Metode:*** *Pada tahap pertama, penelitian terdiri dari 8 kelompok nisin dengan konsentrasi (10-200 mg/mL) dan 3 kelompok nanopartikel $Ca(OH)_2$ konsentrasi (0,425-1,7 mg/mL). Semua kelompok diuji dengan metode difusi terhadap E. faecalis pada hari ke-1 dan ke-5. Bagian kedua, nanopartikel $Ca(OH)_2$ dikombinasikan dengan konsentrasi nisin paling efektif dari penelitian tahap pertama (100 dan 150 mg/mL), kemudian diuji efektivitasnya pada hari ke-1, 3, 7, dan 14. Semua perlakuan diulang sebanyak tiga kali untuk memastikan standarisasi hasil. Analisis statistik dilakukan dengan uji ANOVA dan analisis post hoc (α=0,05).* ***Hasil:*** *Nisin dengan konsentrasi 100 mg/mL dan 150 mg/mL menunjukkan zona hambat terbesar, yaitu 14,33 dan 13,83 mm. Kombinasi nanopartikel $Ca(OH)_2$ dan nisin menghasilkan zona hambat yang lebih kecil di pada pengukuran di hari ke-7 dan 14 dibandingkan dengan nanopartikel $Ca(OH)_2$.* ***Kesimpulan:*** *Nanopartikel $Ca(OH)_2$ dan nisin memiliki efek antimikroba terhadap E. faecalis. Nisin menunjukkan efek antimikroba yang lebih tinggi dibandingkan dengan nanopartikel $Ca(OH)_2$, namun kombinasinya dengan nanopartikel $Ca(OH)_2$ menghasilkan penurunan efektivitas antimikroba pada bakteri E. faecalis.*

## *Kata kunci*

*Antimikroba, E. faecalis, kalsium hidroksida, medikamen saluran akar, nisin*

## INTRODUCTION

Bacterial persistence remains one of the most frequent causes of endodontic treatment failure, primarily due to inadequate root canal disinfection. *Enterococcus faecalis* is the most common bacteria found in cases of root canal treatment failure.[1,2] Its remarkable ability to survive under adverse conditions is attributed to its adaptation to environments with minimal nutrient availability, tolerance to high alkaline pH levels (up to 11.5), and the presence of proton pump systems.[3,4]

Calcium hydroxide ($Ca(OH)_2$) paste is widely used as an intracanal medicament for root canal disinfection agent. $Ca(OH)_2$ acts by releasing hydroxyl and calcium ions; hydroxyl ions diffuse into the dentinal tubules, creating an alkaline environment detrimental to bacterial survival. These ions disrupt bacterial cytoplasmic membranes, protein structures, and DNA. Meanwhile, calcium ions contribute to cell proliferation and the remineralization of hard tissues. Calcium ions act by activating Transforming Growth Factor-beta (TGF-β), which plays a role in biomineralization, and Adenosine Triphosphate (ATP), which facilitates the mineralization process of bone and dentin.[5,6] However, $Ca(OH)_2$ acts slowly, requiring approximately 7–14 days to effectively eliminate microorganisms.[7–9]

Natural limestone material has been successfully synthesized into $Ca(OH)_2$ in nanoparticle size, exhibiting a structure similar to that of commercial $Ca(OH)_2$ commonly used as a root canal medicament in nanoparticle form.[10,11] Nanoparticulate $Ca(OH)_2$ demonstrates superior penetration into the dentinal tubules and complex root canal anatomy. Moreover, $Ca(OH)_2$ nanoparticles exhibit higher antimicrobial efficacy and have been shown to eliminate more bacteria than conventional $Ca(OH)_2$ formulations.[12,13]

Nisin is a bacteriocin produced by Gram-positive bacteria that is odorless, colorless, and tasteless.[14] It exhibits potent antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria in the oral cavity, including *Streptococcus mutans*, *Streptococcus sanguinis*, *Lactobacillus acidophilus*, and *E. faecalis*. The antimicrobial mechanism of nisin is pH-independent and does not induce drug resistance, while maintaining low toxicity. Furthermore, nisin can be used synergistically when combined with conventional therapeutic agents. Studies on *E. faecalis* have demonstrated that nisin possesses greater antimicrobial efficacy than $Ca(OH)_2$, with an optimal concentration of 100 mg/mL for bacterial elimination.[15,16]

Despite the known effectiveness of $Ca(OH)_2$ nanoparticles, their limited efficacy against resistant strains such as *E. faecalis* underscores the need to investigate potential synergistic agents like nisin. The novelty of this study lies in evaluating the time dependent antimicrobial interaction between $Ca(OH)_2$ nanoparticles and nisin, which has not been extensively investigated in previous research. The findings indicate that combining the two agents may reduce their overall effectiveness over time, highlighting the importance of formulation compatibility in developing intracanal medicaments. This study aimed to assess the antimicrobial effectiveness of $Ca(OH)_2$ nanoparticles alone and in combination with nisin against E. faecalis.

## METHODS

This study was designed as a true experimental in-vitro investigation utilizing *E. faecalis* (ATCC 29212) standardized to 0.5 McFarland ($10^8$ CFU/mL) and cultured on Tryptic Soy Agar (TSA) medium. Bacterial identification was confirmed through gram staining and microscopic examination. In the first phase, the antimicrobial efficacy of all experimental groups was evaluated using the agar well diffusion method, by measuring the diameter of the clear inhibition zones. $Ca(OH)_2$ nanoparticles were synthesized following protocols established in previous studies.[12] The nanoparticle suspension was prepared by dissolving $Ca(OH)_2$ in Milli-Q water (Sigma-Aldrich, Germany) at a concentration of 1.7 mg/mL, followed by serial dilutions to obtain concentrations of 0.85 mg/mL and 0.425 mg/mL.[17] A nisin solution (derived from *Lactococcus lactis*, potency ≥900 IU; Sigma-Aldrich, USA) was prepared by dissolving it at a concentration

of 200 mg/mL in Milli-Q water and further diluted gradually to reach a final concentration of 10 mg/mL. [18] Each test solution, comprising various concentrations of $Ca(OH)_2$ nanoparticles and nisin, was introduced into wells on the bacterial culture media. Plates were incubated under anaerobic conditions in anaerobic jar at 37°C. Antimicrobial activity was assessed by measuring inhibition zone diameters on days 1 and 5. Each well created on the agar plate had a standardized diameter of 6 mm and was filled with 50 µL of the respective test solution to ensure consistent diffusion conditions. Days 1 and 5 were selected to evaluate both immediate and short term antimicrobial effects, capturing potential early interactions and sustained activity of the tested agents. A negative control group containing only distilled water was included, as it served as the solvent for all experimental solutions and established the baseline antimicrobial inactivity. All experiments were conducted in triplicate to ensure reproducibility.

In the second phase, $Ca(OH)_2$ nanoparticles were combined with nisin at concentrations that demonstrated the highest antibacterial activity in the initial phase (100 and 150 mg/mL for nisin, and 0.85 mg/mL for $Ca(OH)_2$ nanoparticles). The agar well diffusion method was employed to assess antimicrobial efficacy. A bacterial suspension standardized to 0.5 McFarland was uniformly spread onto Tryptic Soy Agar (TSA) plates, and wells were created using a standardized 6 mm diameter puncher. Each well was filled with 50 µL of the $Ca(OH)_2$ nanoparticle-nisin combination paste(100 mg/mL and 150 mg/mL) and homogenized. A control group consisting of $Ca(OH)_2$ nanoparticles dispersed in distilled water ($Ca(OH)_2$ nanoparticle paste) was included.. Plates were incubated anaerobically at 37°C, and the diameters of inhibition zones were measured with a digital caliper on days 1, 3, 7, and 14.[7,19] These time points were selected based on prior evidence indicating that $Ca(OH)_2$ exhibits peak antimicrobial activity around day 7. Data were tabulated and statistically analyzed using one-way ANOVA with SPSS Statistics 21.0 (IBM Corp.,Armonk, New York, USA), followed by Tukey's post hoc test ($\alpha$=0.05).

## RESULTS

The first phase of the study aimed to determine the concentrations of $Ca(OH)_2$ nanoparticles and nisin that most effectively inhibited the growth of *E. faecalis* on days 1 and 5. The results, presented in Figure 1, show the measurement of the inhibition zone on days 1 and 5 across $Ca(OH)_2$ nanoparticles at concentrations of 0.425, 0.85, and 1.7 mg/mL. At 0.425 mg/mL, the inhibition zone remained relatively stable from day 1 to day 5, whereas 0.85 mg/mL and 1.7 mg/mL showed increased inhibition by day 5. Normality testing using the Shapiro-Wilk test indicated non-normal distribution (p=0.045). However, Levene's test confirmed homogeneity of variances among groups (p=0.173). The Kruskal-Wallis test revealed a significant difference in sensitivity across treatment groups (p=0.017). Post-hoc Tukey analysis showed that $Ca(OH)_2$ 0.85 mg/mL at day 5 differed significantly from $Ca(OH)_2$ 0.425 mg/mL at day 1 and day 5 (p=0.0038), while other comparisons were not statistically significant.

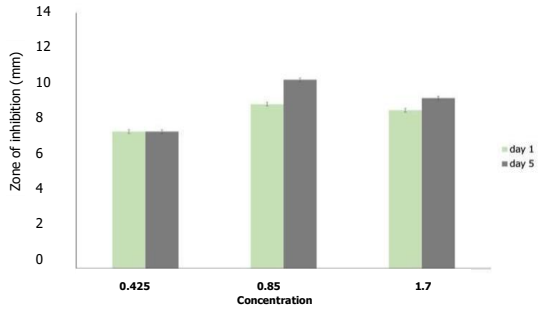


**Figure 1. Zones of inhibition (mm) produced by $Ca(OH)_2$ nanoparticles at 0.425, 0.85, 1.7 mg/mL concentrations on days 1 and 5.**

Figure 2. shows the inhibition zones produced by nisin at various concentrations. The inhibition zones increased with higher nisin concentrations on both day 1 and day 5. On day 1, the bacterial inhibition zones were generally larger than on day 5 across all concentrations, with the difference being more pronounced at lower concentrations (10–30 mg/mL). At higher concentrations (100–200 mg/mL), inhibition zones remained relatively stable between days 1 and 5, suggesting a possible decline in effectiveness of nisin over time that was less evident at high concentrations.

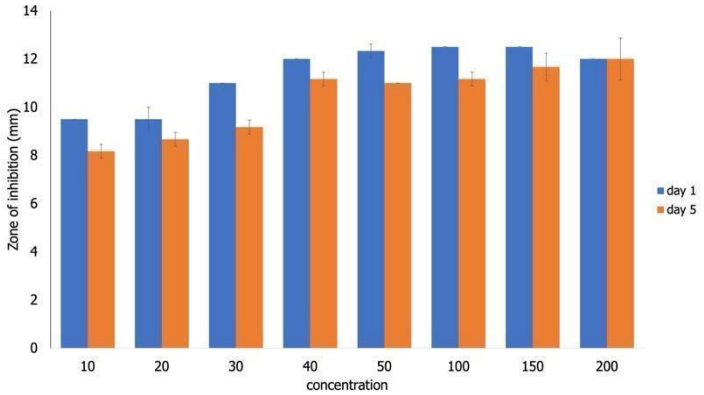


**Figure 2. Zone of inhibition (mm) produced by nisin at concentrations of 10-200 mg/mL after 1 and 5 days of incubation.**

Normality testing using the Shapiro-Wilk test indicated non-normal data distribution (p=0.001), and Levene's test showed unequal variances (p=0.023). The Kruskal-Wallis test identified a significant difference among treatment groups (p=0.001). Post-hoc Bonferroni testing revealed significant differences between lower concentrations (10 mg/mL) at day 5 and higher concentrations (100 and 150 mg/mL) at day 1, indicating greater antimicrobial activity with increasing nisin concentration and shorter exposure time.

The second phase evaluated the antimicrobial efficacy of 0.85 mg/mL $Ca(OH)_2$ nanoparticles used alone (control) and in combination with nisin at two concentrations (100 and 150 mg/mL). Table 1 summarizes the inhibition zone diameters measured on days 1, 3, 7, and 14 across the three treatment groups, (1) $Ca(OH)_2$ nanoparticles alone (control), (2) $Ca(OH)_2$ nanoparticles+nisin (100 mg/mL), and (3) $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL).

**Table 1. Diameter (mm) of zone of inhibition produced by $Ca(OH)_2$ nanoparticles, $Ca(OH)_2$+nisin 100 mg/mL and $Ca(OH)_2$+nisin 150 mg/mL combinations against *E. faecalis***

| | Mean (SD) | | |
|---|---|---|---|
| | **$Ca(OH)_2$ nanoparticles** | **$Ca(OH)_2$ nanoparticles+nisin (100 mg/mL)** | **$Ca(OH)_2$ nanoparticles+nisin (150 mg/mL)** |
| Day 1 | 8.7 (0.29) | 8.7 (0.58) | 9.8 (0.29) |
| Day 3 | 9.0 (0.00) | 8.8 (0.29) | 9.2 (0.29) |
| Day 7 | 11.5 (0.50) | 8.8 (1.04) | 10.0 (0.00) |
| Day 14 | 8.3 (0.58) | 7.7 (0.58) | 6.8 (0.29) |

On day 1, both the control group and the $Ca(OH)_2$ nanoparticles + nisin (100 mg/mL) group exhibited identical inhibition zones (8.7 mm), while the $Ca(OH)_2$ nanoparticles + nisin (150 mg/mL) combination showed significantly greater inhibition (9.8 mm; $p<0.05$), suggesting a possible synergistic effect at the higher nisin concentration. By day 3, inhibition zones were comparable across all treatments (8.8–9.2 mm). On day 7, the control group demonstrated a marked increase in antibacterial activity (11.5 mm), surpassing both combination groups. The $Ca(OH)_2$ nanoparticles + nisin (150 mg/mL) group maintained higher inhibition (10.0 mm) than the $Ca(OH)_2$ nanoparticles + nisin (100 mg/mL) group but remained less effective than the $Ca(OH)_2$ nanoparticles alone. By day 14, antibacterial activity declined in all groups, with the

greatest reduction observed in the $Ca(OH)_2$ nanoparticles + nisin (150 mg/mL) group (6.8 mm). Shapiro-Wilk and Levene's tests confirmed that the data were normally distributed (p=0.649) and had homogeneous variances (p=0.643). One-way ANOVA showed a significant difference in inhibition zones among the treatment groups across incubation times. Tukey's post-hoc analysis identified a significant decrease in antibacterial activity from day 7 to day 14, indicating a time-dependent decline in efficacy, as seen in Table 2.

**Table 2. Tukey's Post-hoc analysis showing intergroup comparison of *E. faecalis* zone of inhibition**

| Study Group | 95% Confidence Interval Value | | p -Value |
|---|---|---|---|
| | Lower Bound | Upper Bound | |
| $Ca(OH)_2$ nanoparticles day 1 vs $Ca(OH)_2$ nanoparticles day 3 | -4.243 | -1.424 | 0.001 |
| $Ca(OH)_2$ nanoparticles day 7 vs $Ca(OH)_2$ nanoparticles day 14 | 1.757 | 4.576 | 0.001 |
| $Ca(OH)_2$ nanoparticles day 14 vs $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 14 | -4.576 | -1.757 | 0.001 |
| $Ca(OH)_2$ nanoparticles day 14 vs $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 7 | 0.924 | 3.743 | 0.001 |
| $Ca(OH)_2$ nanoparticles+nisin (100 mg/mL) day 3 vs $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 14 | 0.424 | 3.243 | 0.004 |
| $Ca(OH)_2$ nanoparticles+nisin (100 mg/mL) day 14 vs $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 14 | -3.743 | -0.924 | 0.001 |
| $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 7 vs $Ca(OH)_2$ nanoparticles+nisin (150 mg/mL) day 14 | -4.576 | -1.757 | 0.001 |

## DISCUSSION

The present findings demonstrate distinct antimicrobial effectiveness for $Ca(OH)_2$ nanoparticles, nisin, and their combinations against *E. faecalis* throughout the observation period (days 1, 3, 7, and 14). In the agar diffusion test, all treatment groups - $Ca(OH)_2$, $Ca(OH)_2$ + nisin 100 mg/mL, and $Ca(OH)_2$ + nisin (150 mg/mL) exhibited peak antimicrobial efficacy at day 7, followed by a measurable decline in activity by day 14 (Table 1).

These results align with the work of Zakaria, et.al[9] who similarly reported the time-dependent antimicrobial activity of $Ca(OH)_2$ against *E. Faecalis*. This pattern is also consistent with previous reports showing that $Ca(OH)_2$ nanoparticle suspensions experience a pH decline after 14 days, thereby reducing their ability to control bacterial growth effectively. $Ca(OH)_2$ nanoparticles exhibit a faster ion dissolution process and reduces the presence of unreacted residual particles, thereby enhancing its overall antimicrobial activity.[20,21] On the contrary, the ability of *E. faecalis* to adapt and proliferate in an alkaline environment over time may limit the effectiveness of $Ca(OH)_2$, as it exerts only a bacteriostatic rather than a bactericidal effect. This finding is consistent with previous studies, which reported a decline in the pH of $Ca(OH)_2$ nanoparticles by day 14, thereby reducing their ability to control bacterial growth effectively.[19]

When the two antimicrobial agents were combined, the inhibition zones were decreased over time (Table 1). This trend suggests that although the freshly prepared combination had appreciable antimicrobial activity, its potency declined with prolonged incubation implying possible neutralization of nisin in the high pH caused by $Ca(OH)_2$ nanoparticles. The antimicrobial mechanism of $Ca(OH)_2$ involves the dissociation of calcium ions, which support tissue remineralization, and hydroxyl ions, which elevate the local pH to inhibit bacterial growth.[22] These results highlight a potential antagonistic interaction between $Ca(OH)_2$ nanoparticles and nisin when used together over extended periods. $Ca(OH)_2$ antimicrobial mechanism relies on a highly alkaline environment to eliminate bacteria. While, nisin exerts its antimicrobial effect independently of the surrounding pH.

However, *E. faecalis* can survive in highly alkaline environments through a proton pump mechanism that maintains intracellular pH homeostasis, limiting the long-term bactericidal effect

of $Ca(OH)_2$.[23] This is advantageous, as *E. faecalis* lacks specific defense mechanisms against such pH independent activity. Nisin acts by penetrating the bacterial cytoplasmic membrane and triggering bacterial murein hydrolase activity, which disrupts essential cellular mechanisms. This results in autolysis and irreversible damage to the bacterial cytoplasmic membrane.[18] $Ca(OH)_2$ nanoparticles produced an increasing inhibition zone over time, with the largest zone observed on day 7, but it declined on day 14.

This pattern suggests a time dependent release of hydroxyl ions that enhances antimicrobial activity.[24] Consistent with the finding of Dianat et al., who reported increased effectiveness of $Ca(OH)_2$ nanoparticles over time due to sustained ion diffusion and penetration into dentinal tubules.[6] In contrast, the combination of $Ca(OH)_2$ nanoparticles+nisin 150 mg/mL showed the highest antimicrobial effect at an early time point, specifically on day 1, which aligns with Shin et al., who reported that nisin exerts rapid bactericidal action through pore formation that produces strong but short-lived antimicrobial effects.[15] This early peak may reflect a temporary synergistic but transient antimicrobial action due to initial pH instability in the $Ca(OH)_2$ nanoparticle and nisin combination.[25] This trend may reflect a common characteristic of certain antimicrobial agents over time.[26]

The antimicrobial effect of $Ca(OH)_2$ nanoparticles observed in this study confirms that reducing the particle size of conventional intracanal medicaments does confer measurable efficacy against *E. faecalis*. Although the inhibition zone of $Ca(OH)_2$ nanoparticles was modest compared with nisin, $Ca(OH)_2$ nanoparticles remain capable of destroying bacterial cell components by the slow release of hydroxyl ions to create a highly alkaline pH. However, $Ca(OH)_2$ nanoparticles are sparingly soluble and diffuse poorly in agar-based assays.[27] Previous studies have reported that even at high concentrations, $Ca(OH)_2$ often produces in minimal zones of inhibition *in vitro*.

Our results align with this, showing only a limited zone of inhibition for $Ca(OH)_2$ nanoparticles, which can attributed to its limited diffusion, as the agar test tends to favor agents that disperse more readily through the medium.[28] Importantly, $Ca(OH)_2$ is expected to greater antimicrobial performance *in situ* than what is suggested entirely by the diameter of the inhibition zone. Due to the nanoparticle size and increased surface area, $Ca(OH)_2$ nanoparticles are capable of penetrating bacterial biofilms and dentinal tubules more effectively than conventional $Ca(OH)_2$ powder. A previous study combining nisin with MTAD, a root canal irrigation material, produced an antimicrobial effect against *E. faecalis*.[27] Nisin is known to exert its antimicrobial effects by disrupting cell wall synthesis and forming pores in the cell membrane resulting in rapid reflux of essential cytoplasmic small molecules.[29] However, in this study, the antimicrobial activity of $Ca(OH)_2$ nanoparticles combined with nisin decreased on days 7, and 14.

This reduction may be attributed to the intrinsic resistance that *E. faecalis* has to $Ca(OH)_2$ nanoparticles so that even if nisin helps more $Ca(OH)_2$ nanoparticles to access the bacteria, $Ca(OH)_2$ itself is unable to sufficient antimicrobial activity. Other factors such as incubation conditions, inoculum, prediffusion, preincubation, and medium thickness may have also influenced the results. Nisin has a low pH (pH 3) so that when combined with $Ca(OH)_2$ it is possible that the pH of $Ca(OH)_2$ nanoparticles is not optimal and affects its effectiveness in eliminating bacteria.[30]

While these findings are derived from *in vitro* data, they indicate that nisin may enhance early phase disinfection in root canal therapy, particularly in cases involving resistant strains.[31] The observed reduction in antimicrobial activity of the $Ca(OH)_2$ nanoparticle and nisin combination over time may be attributed to several factors. One possible explanation is the pH conflict between the two agents, nisin has an acidic pH of approximately 3, which may partially neutralize the strongly alkaline environment (pH 12) required for the optimal antimicrobial action of $Ca(OH)_2$, thereby reducing its efficacy.[31] In addition, prolonged interaction between nisin and the alkaline $Ca(OH)_2$ matrix may compromise the structural stability and bioactivity of nisin, as bacteriocins are known to be pH sensitive and susceptible to degradation under high pH conditions.[32]

Consequently, future research should investigate the efficacy of $Ca(OH)_2$ with nisin treatments whether modifications, such as sequential application or the use of protective carriers, can achieve genuine synergy. This in vitro study used planktonic *E. faecalis*, which may not fully represent biofilm resistance in clinical settings. Furthermore, parameters such as pH changes, chemical stability, and cytotoxicity of the $Ca(OH)_2$ nanoparticle and nisin combination were not evaluated. The study was also limited by the use of a single bacterial strain and a 14-day observation period. Future research should address these limitations by using biofilm models and cell-based assays.

## CONCLUSION

$Ca(OH)_2$ nanoparticles demonstrated peak antimicrobial activity at day 7, reflecting sustained hydroxyl ion release, whereas nisin alone showed superior early antimicrobial action. However, their combination did not exhibit a sustained synergistic effect beyond day 7. The *in vitro* nature of our study implies that factors such as dentin adsorption, tissue buffering, and enzymatic degradation *in vivo* may affect the outcomes. Since *E. faecalis* typically exists within polymicrobial communities, and nisin primarily targets Gram positive bacteria, it may not be effective against Gram negative or fungal species commonly associated with persistent infections.

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