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# Comparative antimicrobial efficacy of chitosan and a high-potency papain gel (Brix 3000) as endodontic irrigants in deciduous teeth: an in vitro analysis

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**Title Page**

# Comparative Antimicrobial Efficacy of Chitosan and a High-Potency Papain Gel (Brix 3000) as Endodontic Irrigants in Deciduous Teeth: An In Vitro Analysis

**RUNNING TITLE:** Chitosan vs Papain as Endodontic Irrigants

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**DECLARATIONS**

Authors' contributions

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Ethics approval and consent to participate: Ethical approval for this study was obtained from the Erciyes University Non-Invasive Clinical Research Ethics Committee (Approval No: 2021/603). All methods involving extracted deciduous teeth were performed in accordance with the relevant guidelines and regulations. Written informed consent for the use of the extracted teeth was obtained from the parents/legal guardians. For children aged 2–8 years, the study information was read aloud and explained in age-appropriate language, and age-appropriate assent was obtained without a separate written assent form. This study was not a clinical trial; therefore, clinical trial registration was not required.
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**Abstract**

**Background:** In cases of irreversible pulpitis, root canal treatment is preferred over extraction. The success of endodontic therapy depends on chemical irrigation in addition to mechanical preparation. Sodium hypochlorite, although widely used, has disadvantages, including unpleasant taste and odor and potential toxic effects on periapical tissues and developing permanent tooth germs. Therefore, research on alternative irrigating solutions is ongoing. The aim of this study was to comparatively evaluate the antimicrobial efficacy of chitosan and a high-potency papain-based gel (Brix 3000) compared with sodium hypochlorite in deciduous teeth.

**Methods:** In this study, thirty-two extracted anterior deciduous teeth were standardized to a root length of 12 mm, instrumented to size F3, and inoculated with *E. faecalis* to establish an early-stage colonization model. The specimens were randomly assigned to three experimental groups and positive/negative control subgroups. The experimental groups received 0.2 g/100 mL (0.2% w/v) chitosan solution (prepared in 0.5%

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v/v acetic acid), 1% (w/v) papain solution prepared from Brix 3000 in distilled water, and 2.5% sodium hypochlorite. The antimicrobial efficacy was assessed in terms of colony-forming units (CFU), and statistical analysis was performed. A p value of $< 0.05$ was considered statistically significant for all tests.

**Results:** A statistically significant difference was observed among the groups regarding *E. faecalis* counts ($p < 0.001$). The papain group showed significantly higher $\log_{10}$(CFU/mL) values ($3.10 \pm 1.57$) compared with the chitosan ($0.11 \pm 0.17$; $p = 0.010$), sodium hypochlorite ($0.0 \pm 0.0$; $p = 0.008$), and negative control ($0.0 \pm 0.0$; $p = 0.008$) groups. No significant difference was found between the chitosan and sodium hypochlorite groups ($p > 0.05$).

**Conclusion:** Within the limitations of this in vitro study, chitosan showed a marked reduction in viable bacterial counts against early-stage E. faecalis colonization in deciduous teeth under the present experimental conditions, whereas the papain-based formulation showed reduced antibacterial performance. However, these findings should not be interpreted as clinical equivalence to sodium hypochlorite. Further studies using mature multispecies biofilm models, clinically relevant irrigation times, and biocompatibility assessments are required before clinical recommendations can be made.

**Keywords:** Chitosan, Papain, Deciduous teeth, Root canal treatment,

## 1.BACKGROUND

Microorganisms and the biofilm structures they form play a fundamental role in the development of root canal infections. Particularly in deciduous teeth, the anatomical complexity of the root canal system makes the complete elimination of microorganisms difficult and may limit the success of endodontic treatment. **(1)** Therefore, mechanical preparation must be supported by chemical irrigation aimed at removing bacterial biofilm and residual microorganisms. The antibacterial efficacy of irrigating solutions is of critical importance for the success of endodontic therapy. **(2)**

Enterococcus faecalis (*E. faecalis*), which is frequently isolated in endodontic infections, is particularly associated with failed root canal treatments due to its facultative Gram-positive anaerobic nature, resistance to adverse environmental conditions, and ability to form biofilms. These characteristics have made *E. faecalis* a widely used reference microorganism in evaluating the antibacterial efficacy of endodontic irrigating solutions. **(3,4)** Today, sodium hypochlorite is considered the most commonly used irrigating agent in root canal irrigation because of its antiseptic and tissue-dissolving properties. **(5,6)** However, its unpleasant taste and odor, as well as its potential toxic effects on periapical tissues and on the developing permanent tooth germs underlying deciduous teeth, raise certain concerns regarding its use, particularly in pediatric dentistry. **(5)**

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Due to these limitations, interest in natural and alternative irrigating agents has increased. Chitosan and papain are natural substances evaluated in endodontics due to their distinct antimicrobial efficacy and mechanisms of action. Chitosan interacts with the bacterial cell wall, while papain, through its proteolytic activity, acts on organic tissues and biofilm structures. **(6–8)** However, the antimicrobial efficacy of plant-based agents has been reported to be variable and often limited compared with sodium hypochlorite. **(9)** While the antimicrobial potential of Brix 3000 has been documented in the context of chemomechanical caries removal **(10)**, research exploring its application as a root canal irrigant against intraradicular bacterial colonization remains relatively limited. Although recent studies on papain-based formulations have shown promising results against *E. faecalis*, data specifically focused on the high-potency (30,000 U/mg) Brix 3000 formulation in deciduous teeth is still emerging.

This study was designed with a multi-stage objective to evaluate the antimicrobial efficacy of natural-origin irrigants in pediatric endodontics. The primary objective was to comparatively evaluate the antimicrobial efficacy of 0.2% chitosan and a high-potency papain-based formulation (Brix 3000, 30,000 U/mg) against early-stage *E. faecalis* colonization in deciduous teeth**,** using 2.5% sodium hypochlorite as the gold standard. Furthermore, the secondary objectives included determining the Minimum Inhibitory Concentration of the experimental agents to validate the concentrations employed in the root canal model and performing solvent validation to ensure that the observed antimicrobial effects were attributed to the active agents (chitosan and papain) rather than the solvents (0.5% acetic acid and distilled water).

The null hypothesis ($H_{0)}$ tested was that there would be no significant difference in the antimicrobial efficacy among 2.5% NaOCl, 0.2% chitosan, and 1% papain-based Brix 3000. The alternative hypothesis ($H_{1)}$ was that at least one of the experimental irrigants would demonstrate a significantly different reduction in *E. faecalis* colony-forming units (CFU/mL).

## 2. METHODS

### 2.1 Ethical Approval & Sample size

Ethical approval for the study was obtained from the Erciyes University Clinical Research Ethics Committee (2021/603). All methods were performed in accordance with the relevant guidelines and regulations. The experimental procedures were conducted between March 2024 and June 2024.

Based on the effect size reported by Keskin et al. **(11)** (effect size = 0.8), with an alpha significance level of 0.05 and 95% power, the required sample size was calculated as 8 anterior deciduous teeth per group, for a total of 32 teeth.

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The study included 32 specimens allocated to three experimental groups (n = 8 each) and two control groups (n = 4 each). The positive control specimens were inoculated with *E. faecalis* but received no irrigation treatment, whereas the negative control specimens were neither inoculated nor treated, to confirm the validity and sterility of the experimental model.

Prior to the study, written informed consent for the use of the extracted teeth was obtained from the parents/legal guardians. The children were aged 2–8 years; the study was explained to them in age-appropriate language, and their assent was obtained without a separate written assent form. The teeth were prospectively collected following clinically indicated extractions that were determined independently of the research and were not selected from a pre-existing pool.

**2.2 Inclusion criteria for the teeth**

Maxillary deciduous anterior teeth with less than one-third physiologic root resorption, free of cracks or fractures, with no previous endodontic treatment, no caries extending to the root surface, and a root canal length of at least 12 mm were included in the study.

Teeth presenting advanced internal or external resorption, marked canal calcification, severe anatomical anomalies or caries extending to the root surface on radiographic evaluation were excluded. Although a certain degree of anatomical heterogeneity is inevitable due to the physiological characteristics of deciduous teeth, all samples were radiographically screened prior to inclusion in order to minimize this variability.

The teeth had been extracted for orthodontic reasons or due to clinical necessity, such as prolonged retention, and teeth showing signs suggestive of inflammatory resorption were excluded.

**2.3 Preparation of the samples**

Soft tissue remnants and debris on the teeth that met the inclusion criteria were removed using a curette, and the teeth were stored in 0.1% thymol solution at room temperature until use. The coronal portions of the samples were removed under water cooling using a diamond disc (SH 393190, Sunshine Diamonds, Langenhagen, Germany) to obtain a standardized root length of 12 mm in all teeth. Root length standardization was confirmed using a digital caliper.

The working length was determined 1 mm short of the apical foramen using a #15 K-type file (Shenzhen Denco Medical, Shenzhen, China), and the apical foramen was sealed with a flowable composite resin (i-

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Flow, i-Dental, Siauliai, Lithuania) to prevent leakage during irrigation. Coronal enlargement was performed using Gates–Glidden drills sizes 1 and 2 (Shenzhen Perfect Medical, Ankara, Türkiye).

Root canal preparation was performed using ProTaper Gold finishing files F1 (#20/.07), F2 (#25/.08), and F3 (#30/.09) in a sequential manner. The files were operated with an X-Smart Plus endodontic motor (Dentsply Sirona, Ballaigues, Switzerland) at a rotational speed of 300 rpm and a torque setting of 2.0 Ncm, following the manufacturer's instructions. The same instrumentation sequence and motor settings were used for all specimens to ensure reproducibility.

During instrumentation, the root canals were irrigated with 2 mL of sterile saline after each instrument change. Upon completion of instrumentation, the canals were sequentially irrigated with 17% EDTA, sterile saline, and 2.5% NaOCl. Each solution was applied separately for 3 minutes (total 9 minutes) using a volume of 2.5 mL to remove residual debris and the smear layer as part of the standardized pre-experimental cleaning procedure. Since irrigant agitation may influence debris removal and antimicrobial efficacy, no manual, sonic, or ultrasonic agitation was performed at this stage in order to avoid introducing an additional procedural variable during specimen preparation **(12)**. A final irrigation with 5 mL sterile saline was performed in all samples. Following the final irrigation, the canals were dried with paper points and sterilized in an autoclave at 121°C under 15 psi pressure for 60 minutes prior to the experimental procedures.

A schematic summary of the sample preparation and canal shaping procedures is presented in Figure 1.

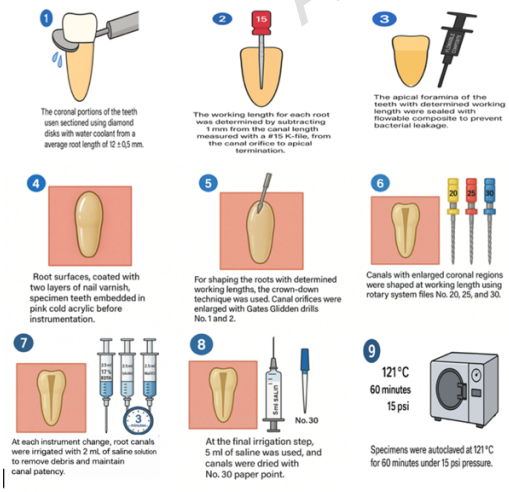



**Figure 1:** Step by step demonstration of the preparation procedure for extracted deciduous teeth

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**2.4 E. faecalis Infection Model**

The ATCC (American Type Culture Collection) 29212 strain of *E. faecalis* was used to infect the root canals. The bacterial strain was initially revived from a frozen stock maintained at -80°C by inoculating it into 10 mL of sterile Brain Heart Infusion (BHI) broth and incubating it at 37°C for 24 hours under aerobic conditions to ensure viability. Following this initial revival, the purity of the culture was confirmed by streaking it onto BHI agar plates to obtain isolated, grown colonies. Brain Heart Infusion (BHI) agar was prepared by dissolving 34 g of medium in 1 L of distilled water. The medium was shaken until homogeneous, sterilized in an autoclave at 121°C for 30 minutes, cooled to 45–50°C, and poured into Petri dishes. The prepared plates were incubated aerobically at 37°C for 24 hours.

The grown colonies were transferred into 10 mL of Brain Heart Infusion Broth (BHI). The bacterial suspension was standardized to a 1.0 McFarland turbidity standard, corresponding to approximately $3 \times 10^8$ CFU/mL**,** using a digital densitometer **(**DEN-1 McFarland Densitometer, Grant Instruments, Cambridge, UK). This standardized bacterial suspension in BHI broth was used directly for the inoculation of the root canals to ensure the immediate interaction of a known concentration of viable cells with the dentinal walls. To ensure accuracy, the bacterial concentration was verified by serial dilution and colony-forming unit counting on BHI agar after 24 hours of incubation.

The sterilized samples were placed into wells under a sterile cabinet to prevent contamination. The prepared tooth roots were numbered and placed into Eppendorf tubes, and 10 µL of the *E. faecalis* culture was inoculated into each root canal using a micropipette. The tubes were incubated at 37°C for 24 hours. In this 24-hour early-colonization model, fresh culture medium was not added and reinoculation was not performed during the incubation period.

The infection model used in this study was designed as a standardized early colonization model to evaluate the immediate antibacterial effect of the experimental irrigants under controlled in vitro conditions. While it is acknowledged that *E. faecalis* is frequently associated with persistent endodontic infections and later-stage bacterial colonization, mature *E. faecalis* biofilms typically require longer maturation periods, often ranging from 7 to 21 days, to develop complex structural resistance. Therefore, the 24-hour incubation period was intentionally selected for primary screening rather than to reproduce a mature, structurally complex biofilm model**.** Short-duration *E. faecalis* infection models have also been used in previous experimental studies involving primary teeth, although bacterial exposure and culture-maintenance protocols have varied across studies **[3,13].** Accordingly, the present model should be interpreted as an early-colonization screening model, providing a controlled setting for evaluating the initial antibacterial

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activity of the tested agents rather than representing a mature clinical biofilm. Successful canal contamination was verified by bacterial growth in the infected untreated positive control specimens, whereas the negative control specimens showed no bacterial growth, confirming both successful inoculation and maintenance of sterility.

**2.5 Preparation of Chitosan and Papain Solutions & Determination of MIC Values**

While preparing the chitosan-containing irrigating solution, the methodology described by Sarkees et al. was followed **(14).** The chitosan solution was prepared by dissolving 0.2 g of chitosan in 100 mL of 0.5% acetic acid to obtain a 0.2% (w/v) concentration. The antimicrobial activity of the chitosan preparation was confirmed by MIC testing under broth microdilution conditions. The final 0.2% (w/v) concentration used in the root canal model was consistent with previous endodontic studies reporting antibacterial activity of 0.2% chitosan against *E. faecalis* **(15,16).** A 1% concentration of acetic acid was not preferred because it showed independent antibacterial activity in the preliminary solvent validation performed in the present study. Due to the low solubility of chitosan, a magnetic stirrer was used for 2 hours to obtain a homogeneous and clear solution. The antibacterial activity of 0.5% acetic acid was also evaluated separately as a solvent control.

For the papain-based irrigant, the commercially available Brix 3000 gel, containing 10% papain, was diluted 10-fold in distilled water at a 1:10 (w/v) ratio to obtain a 1% (w/v) working suspension. Although Brix 3000 is originally supplied as a high-viscosity gel for chemomechanical caries removal, dilution was performed in the present study to obtain a fluid preparation suitable for standardized intracanal delivery as an experimental endodontic irrigant. This modification from the original gel form to a fluid working suspension was made to allow reproducible and standardized intracanal delivery under the present experimental irrigation protocol. Due to the high viscosity of the carbomer-based gel, the mixture was subjected to mechanical agitation using a vortex mixer for 60 seconds to achieve a macroscopically homogeneous suspension. No additional filtration or centrifugation was performed, as visual inspection confirmed uniform dispersion of the gel within the solvent. To minimize potential enzymatic degradation, the papain-based preparation was prepared freshly immediately before each experimental procedure and used within a short time frame. Because papain is an enzyme, its activity may be influenced by factors such as storage conditions, temperature, pH, and prolonged exposure to aqueous media. Potential loss of enzymatic activity may be minimized through fresh preparation, appropriate storage conditions, stabilization strategies, or formulation approaches designed to preserve enzyme activity until use. The same commercial product batch, dilution ratio, solvent, mixing time, and preparation procedure were used for all specimens to ensure consistency of the applied preparation.

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A broth microdilution test was used to determine the minimum inhibitory concentration (MIC) of the solutions and of the acetic acid used in their preparation against *E. faecalis* colonies. The solutions were placed into a 96-well ELISA plate, and a two-fold serial dilution was performed. (Figure 2)

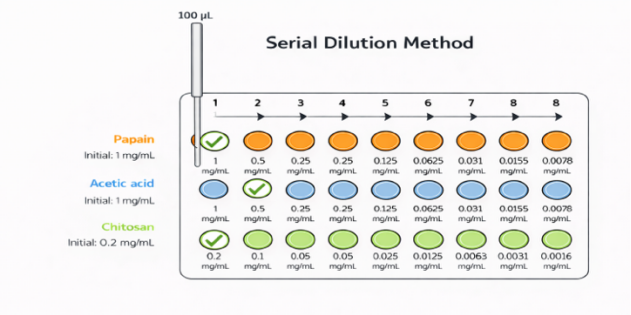



**Figure 2:** Serial dilution method used to determine the minimum inhibitory concentration (MIC) of papain, acetic acid, and chitosan against *Enterococcus faecalis*.

Bacterial suspension was added to the diluted solutions, and bacterial growth was allowed at 37°C for 24 hours. The prepared samples were then transferred to Brain Heart Infusion Broth medium and incubated at 37°C for an additional 24 hours. The antibacterial efficacy of the solutions at different concentrations was evaluated.

The MIC values obtained in the present study were used to confirm the antimicrobial activity of the tested preparations under broth microdilution conditions. However, MIC testing was used only to confirm antimicrobial activity and was not intended to provide physicochemical characterization of the diluted Brix 3000 preparation. The final irrigation concentrations (0.2% for chitosan and 1% for papain-based Brix 3000) were selected for use in the standardized root canal model, taking into account potential dentin buffering effects and diffusion limitations.

### 2.6 Application of Irrigation Solutions

The irrigation solutions were sterilized under UV light prior to use to prevent the risk of contamination. Following *E. faecalis* colonization, the previously numbered specimens were randomly assigned to three experimental groups using a computer-generated random number table. The positive control specimens were inoculated but left untreated, whereas the negative control specimens were not inoculated and received no irrigation treatment.

The 2.5% sodium hypochlorite solution, 0.2 g/100 mL (0.2% w/v) chitosan solution (in 0.5% v/v acetic acid), and 1% (w/v) papain-based Brix 3000 suspension prepared in distilled water were sterilized under

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UV light in a closed cabinet prior to use to prevent contamination. For each specimen, a total of 6 mL of the irrigation solution was delivered into the root canals for 2 minutes using a conventional needle syringe. Following this initial delivery, the solutions were allowed to remain in passive contact with the root canal walls for 30 minutes.

The 30-minute passive contact period was selected as a standardized experimental screening duration to compare the antimicrobial performance of the tested agents under controlled laboratory conditions. This duration was based on previous in vitro studies in which similar contact periods were used to evaluate the antimicrobial activity of alternative, natural-origin, or enzymatic endodontic irrigants against *E. faecalis* (**15,17,18).** The same irrigation volume, delivery time, passive contact period, and removal procedure were applied to all experimental groups.

During this period, the specimens were kept in a vertical position within sterile Eppendorf tubes to ensure that the irrigation solutions remained inside the root canals. At the end of the contact time, the root canals were rinsed with distilled water, and the irrigation solutions were mechanically removed.

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**2.7 Bacterial Sampling**

To improve the standardized recovery of bacteria adherent to the root canal surface, the canal was first rewetted with sterile saline. A size 30 H-type file was inserted to the working length, and mechanical scraping was performed using 20 standardized longitudinal movements along the root canal walls over 1 minute while saline was present in the canal**.** Subsequently, multiple sterile size 30 paper points were placed into the root canals and left in situ for 30 seconds to allow absorption of the canal contents containing the detached bacterial material**.** The paper points were then transferred into Eppendorf tubes containing 1 mL of sterile saline, labeled according to the group numbers of the teeth. To facilitate the transfer of bacterial content from the paper points into the saline solution, the tubes were vortexed for 20 seconds.

**2.8 Colony Counting and CFU Evaluation**

The samples in the Eppendorf tubes were serially diluted in sterile saline to obtain diluted solutions. From each prepared solution, 0.1 mL was inoculated onto Brain Heart Infusion Agar using a micropipette and evenly spread over the surface with a sterile plastic Drigalski spatula. The Petri dishes were incubated at 37°C for 24–48 hours. Following incubation, the resulting bacterial colonies were manually counted, and the results were calculated as colony-forming units per milliliter (CFU/mL).

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Before statistical analysis, bacterial colony counts (CFU/mL) were subjected to $\log_{10}$(CFU/mL) transformation to approximate normal distribution. In samples with no observed colony growth (CFU = 0), 1 was added to the CFU values prior to logarithmic transformation [$\log_{10}$(CFU + 1)] to avoid mathematical indeterminacy, in accordance with commonly used approaches in the literature. All statistical analyses were performed using the log-transformed data.

### 2.9 Statistical Analysis

Statistical analyses were performed using SPSS version 23.0. The normality of the data distribution was assessed using the Shapiro–Wilk test. As the assumptions for parametric analysis were not met for the comparison of log-transformed CFU values among the study groups, the Kruskal–Wallis test was used for the overall group comparison**.** Descriptive statistics were presented as minimum, first quartile (Q1), median, third quartile (Q3), and maximum values. A p-value of $< 0.05$ was considered statistically significant.

## 3. RESULTS

After 24 hours of incubation, the bacterial colonies formed on the Petri dishes were examined (Figure 3), and the number of *E. faecalis* colonies was quantified as colony-forming units per milliliter (CFU/mL). For statistical analyses, the data were $\log_{10}$- transformed and presented as $\log_{10}$(CFU/mL).

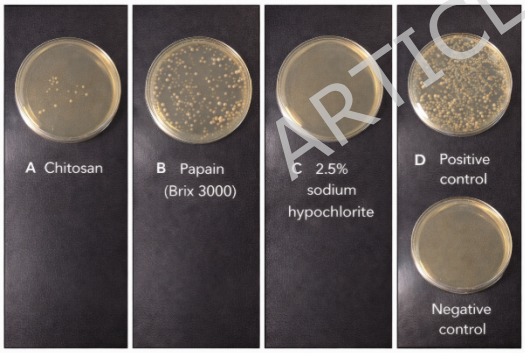



**Figure 3:** Representative images of *Enterococcus faecalis* colony growth in the study groups: chitosan (A), papain (Brix 3000) (B), 2.5% sodium hypochlorite (C), and control groups (D; positive and negative).

No bacterial growth was observed in the sodium hypochlorite group or in the non-infected negative control group. Among the remaining groups, the mean $\log_{10}$(CFU/mL) values were $0.11 \pm 0.17$ for the chitosan group and $3.10 \pm 1.57$ for the papain group. In the infected but untreated positive control group, the mean value was $7.57 \pm 0.67$. The distribution of $\log_{10}$(CFU/mL) values is presented in Table 1.

| Group | Median (Q1-Q3) | Range |
|---|---|---|

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| | | |
|---|---|---|
| Chitosan | 0.00 (0.00-0.20) | 0.00-0.40 |
| Papain | 3.20 (2.40-4.30) | 1.50-5.10 |
| Sodium hypochlorite | 0.00 (0.00-0.00) | 0.00-0.00 |
| Positive control | 4.00 (1.80-6.50) | 0.80-8.40 |

**Table 1:** Distribution of $\log_{10}$(CFU/mL) values of *Enterococcus faecalis* among study groups

Data are presented as median (first quartile- third quartile) and range (Table 1). The sodium hypochlorite and negative control groups demonstrated complete elimination of bacterial growth, whereas the papain group showed higher residual bacterial counts. The positive control group exhibited the highest bacterial load, confirming the successful establishment of the infection model.

A statistically significant difference was observed among the groups ($p < 0.001$), as illustrated in Figure 4. The papain group showed significantly higher bacterial counts compared with the chitosan ($p=0.010$) and the sodium hypochlorite groups ($p=0.008$). No statistically significant difference was found between the chitosan and sodium hypochlorite groups ($p > 0.05$).

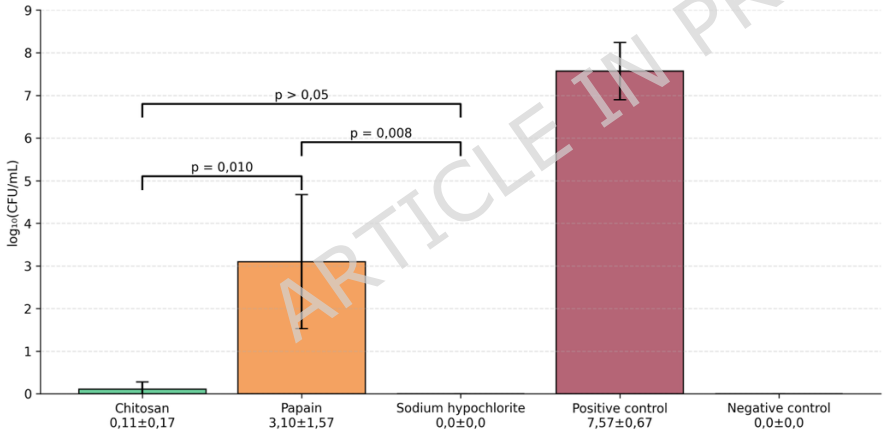



**Figure 4:** $\text{Log}_{10}$(CFU/mL) values of *Enterococcus faecalis* among study groups

## 4. DISCUSSION

In this study, the antibacterial efficacy of chitosan and a papain-containing Brix 3000 formulation used for root canal irrigation in deciduous teeth was comparatively evaluated against 2.5% sodium hypochlorite on *Enterococcus faecalis*. No statistically significant difference was detected between the chitosan and sodium hypochlorite groups under the present experimental conditions; however, the papain-containing Brix 3000 formulation showed lower antibacterial performance than both agents. Therefore, the null hypothesis was rejected, as significant differences in antibacterial efficacy were observed among the tested irrigants, particularly for the papain-based Brix 3000 formulation compared with chitosan and sodium hypochlorite.

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Enterococcus faecalis is frequently associated with persistent endodontic infections and has been isolated in deciduous teeth with persistent endodontic infections and failed pulpectomy cases. Although primary root canal infections are polymicrobial in nature, *E. faecalis* is commonly used as a reference microorganism in experimental endodontic studies due to its resistance to harsh environmental conditions and its ability to survive intracanal procedures. Therefore, the present monospecies model was selected to provide a standardized and reproducible platform for comparing the antibacterial efficacy of different irrigants. Nevertheless, it should be acknowledged that this simplified model representing early-stage bacterial colonization does not fully represent the complex and mature multispecies biofilm found in clinical conditions. Although standardized inoculation procedures were applied, the absence of baseline bacterial quantification for each specimen limits the ability to confirm equal initial bacterial loads among groups. This factor should be considered when interpreting intergroup variability and the magnitude of antibacterial effects. Therefore, the findings of the present study should be interpreted as an initial screening of antimicrobial potential rather than a direct representation of clinical endodontic infections involving mature multispecies biofilms. **(3,7,10,19)**

Short-duration *E. faecalis* infection models have also been used in previous experimental studies involving primary teeth, although the duration of bacterial exposure and culture-maintenance protocols have varied substantially across studies (3,13). These reports support the use of short-term models for preliminary antimicrobial screening; however, they do not substitute for mature multispecies biofilm models when clinical relevance is the primary objective. Accordingly, the present findings should be interpreted within the context of early-stage bacterial colonization. **(3,13)**

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Sodium hypochlorite is frequently preferred in root canal irrigation due to its strong antibacterial effect. **(20)** The literature has reported that a 2.5% concentration provides effective antibacterial activity. **(21)** For this reason, in the present study, the efficacy of natural-origin irrigating agents was compared with 2.5% sodium hypochlorite as the reference agent. While higher concentrations of NaOCl (e.g., 5.25%) are available, the 2.5% concentration was selected because it is commonly used in pediatric endodontic studies and clinical practice. This concentration was considered an appropriate reference for comparison while limiting the potential for chemical injury to the periapical tissues of deciduous teeth **(22).**

The low residual bacterial counts observed in the chitosan group under the present experimental conditions are noteworthy; however, the absence of a statistically significant difference from sodium hypochlorite should not be interpreted as evidence of equivalence. The 0.2% chitosan concentration used in the present study should not be regarded as an arbitrarily weak formulation; rather, it represents an established experimental concentration that is consistent with previous endodontic studies reporting antibacterial

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activity against *E. faecalis* **(15,16)**. In addition to its antibacterial potential, previous studies have suggested that 0.2% chitosan may exert a more conservative effect on root dentin than conventional chelating agents, with less reduction in dentin microhardness and more favorable surface characteristics compared with 17% EDTA **(23,24)**. Taken together, these findings support the interpretation of 0.2% chitosan as a concentration that may balance antibacterial activity with preservation of dentin surface characteristics. However, because dentin microhardness and surface roughness were not directly evaluated in the present study, this interpretation should be considered as supporting literature-based background rather than a direct experimental outcome of the present study.

The selection of a 30-minute passive contact period served as a standardized experimental screening protocol to evaluate the antimicrobial potential of these novel agents. This approach is relevant for determining whether these bio-based materials show antibacterial potential under controlled in vitro conditions. Previous literature supports that while sodium hypochlorite exerts rapid action, alternative agents like chitosan require sufficient contact time to optimize their electrostatic interaction with bacterial cell walls and enhance antibacterial activity against adherent *E. faecalis* **(16)**. Similarly, the enzymatic activity of papain-based formulations depends on adequate time for proteolytic degradation of the protein matrix and disruption of early bacterial colonization within the root canal microenvironment **(17)**. However, the 30 minute passive contact period exceeds typical chairside irrigation times in pediatric dentistry and therefore represents a practical limitation of the present experimental protocol. The antibacterial performance observed under this prolonged exposure should not be directly extrapolated to routine clinical use. Future studies should evaluate whether comparable antibacterial effects can be achieved using shorter and more clinically applicable contact times.

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Although no statistically significant difference was detected between the chitosan and sodium hypochlorite groups ($p > 0.05$), complete elimination was observed only in the sodium hypochlorite group, whereas low residual bacterial counts remained in some chitosan specimens. Therefore, the distinction between statistical significance and clinical relevance should be interpreted cautiously when evaluating these findings. This observation aligns with the findings of Cakici et al., whose meta-analysis indicated that sodium hypochlorite often demonstrates higher antibacterial efficacy specifically in studies utilizing bacterial culturing methods. However, they also noted that such results might vary when alternative detection methods are used, suggesting that the antimicrobial potential of chitosan might be interpreted differently depending on the assessment method. **(25)**

In deciduous teeth, physiological root resorption and increased apical foramen diameter may increase the risk of extrusion of irrigating solutions beyond the root canal. In addition, the irritant properties of sodium

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hypochlorite, as well as its unpleasant taste and odor, may negatively affect cooperation in pediatric patients. **(11,14,19,20,25,26)** Considering these clinical disadvantages, natural-origin alternative agents have gained importance. **(20,21,25,27,28)** In the present study, the antibacterial effect of chitosan under the present experimental conditions may therefore justify further investigation under more clinically representative experimental conditions. The majority of studies evaluating the antibacterial efficacy of chitosan have been conducted in permanent teeth. Goel et al. **(4)**, Jaiswal et al. **(16)**, Enggardipta et al. **(20)**, and Kadour et al. **(28)** reported results similar to those of sodium hypochlorite. The findings of the present study are consistent with these reports and support the further investigation of chitosan-based irrigants in deciduous teeth as well.

Furthermore, a recent systematic review and meta-analysis evaluating the antimicrobial efficacy of chitosan against *E. faecalis* concluded that chitosan and its nanoparticles can be considered a viable alternative to sodium hypochlorite. Interestingly, the meta-analysis revealed that while sodium hypochlorite appeared statistically more effective in studies using bacterial culture methods, chitosan showed superior performance when evaluated through confocal laser scanning microscopy. This distinction underscores the influence of the evaluation methodology on the reported efficacy of irrigants. **(25)**

Although most studies in the literature report may suggest a potential clinical difference between chitosan and sodium hypochlorite in terms of antibacterial efficacy, variable results have been observed regarding the number of bacteria eliminated. **(16,27–29)** Previous studies have generally prepared chitosan solutions in 1% acetic acid and at higher concentrations (0.3 g). **(16,28,29)** Such formulation differences may be one of the factors explaining the variability in the reported levels of antibacterial activity.

In the present study, 0.5% acetic acid was preferred based on MIC analysis in order to minimize the potential antibacterial contribution of acetic acid. This approach aimed to evaluate the antibacterial effect of chitosan in a more isolated manner. The findings demonstrated no statistically significant difference between chitosan and sodium hypochlorite, and these results are consistent with those reported by Faiek et al. **(29)** and Bastaway et al. **(30)** in deciduous teeth.

Lopez-Flores et al. **(21)** demonstrated that antibiotic-loaded chitosan nanoparticles showed strong antibacterial activity against *E. faecalis*. These findings further support the increasing interest in biopolymer-based and biologically derived agents in endodontic disinfection, aligning with the rationale for investigating enzymatic agents such as papain. Papain is widely used in dentistry, particularly as a chemomechanical caries removal agent, due to its proteolytic properties similar to those of the human pepsin enzyme. Its antibacterial effect has been reported to be associated with the chloramine components

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in its formulation. The use of papain-containing materials as root canal irrigants has been evaluated in a limited number of studies, in which different papain formulations such as Papacarie and papain gel were investigated. **(31–33)**

However, no study has been identified in the literature evaluating the antibacterial efficacy of Brix 3000, a high-papain-content commercial formulation, as a root canal irrigant. Investigating Brix 3000 in this context addresses a significant knowledge gap, as its documented use has primarily been restricted to chemomechanical caries removal. The selection of Brix 3000 as the papain source, rather than a pure laboratory-grade enzyme, was based on its commercially available stabilized formulation and enzyme bio-encapsulation technology, which has been reported to provide superior enzyme stability and high proteolytic activity of 30,000 U/mg **(34)**. Therefore, evaluating Brix 3000 may provide formulation-specific information beyond that obtained from testing papain as an isolated enzyme alone. In the present study, the commercial Brix 3000 gel was diluted 10-fold to obtain a 1% papain-based working suspension. This concentration was selected to convert the original high-viscosity gel into a reproducible fluid preparation suitable for standardized intracanal delivery, while also allowing evaluation of papain at a concentration range previously reported to exhibit antibacterial and antibiofilm activity with favorable biological characteristics **(17,35)**. Similar methodological adaptations have also been reported in studies evaluating gel-based or papain-containing agents, such as Carisolv, Papacarie, and other enzymatic formulations, for intracanal disinfection or root canal irrigation **(17,36)**. Moreover, previous studies suggest that the antimicrobial performance of endodontic disinfectants is influenced not only by the active agent concentration, but also by formulation type, delivery form, chemical interactions, and experimental conditions **(36,37).** Accordingly, the findings of the present study should be interpreted as the antibacterial performance of the 1% papain-based Brix 3000 working suspension under the present experimental protocol, rather than as the clinical performance of the original undiluted gel formulation. These findings provide preliminary information for future studies aimed at optimizing papain-based formulations specifically designed for endodontic irrigation.

Different findings have been reported in the literature regarding the antibacterial efficacy of papain-containing agents in the root canal system. Singhal et al. compared the root canal cleaning efficacy of Papacarie and sodium hypochlorite in deciduous teeth and reported similar results between the two agents **(31)**. Maru et al. **(32)** and Prasad et al. **(8)** also demonstrated a significant reduction in colony counts following irrigation with papain-containing agents. These studies suggest that papain may have potential antibacterial activity.

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In the present study, although the papain-containing Brix 3000 group showed a significant reduction in bacterial counts compared to the positive control group, it demonstrated higher $\log_{10}$(CFU/mL) values than the sodium hypochlorite and chitosan groups. This finding is consistent with studies reporting lower antibacterial efficacy of papain compared to sodium hypochlorite. Despite its high papain concentration, the limited antibacterial performance of Brix 3000 as an irrigating solution suggests that the effectiveness of papain-based agents in the root canal environment may not depend solely on concentration. These findings may provide a basis for future studies evaluating shorter, clinically applicable irrigation protocols under conditions that more closely simulate the clinical environment.

The selection of 0.2% chitosan and 1% papain-based Brix 3000 was intended to evaluate these agents at experimentally applicable concentrations that were supported by previous in vitro studies and suitable for standardized intracanal delivery under the present laboratory protocol **(15,16).** Additionally, the use of manual needle irrigation without supplemental activation protocols was intended to isolate and evaluate the standalone chemical efficacy of these novel agents, ensuring that the results reflect their inherent antimicrobial potential without the confounding mechanical influence of agitation techniques **(22)**.

**Limitations**

As this study was designed as an in vitro investigation, it may not fully reflect clinical conditions. Factors such as the complex multispecies biofilm structure of the root canal system and host response could not be evaluated within this model.

The absence of pre-treatment bacterial counts for each specimen does not allow direct confirmation of the initial bacterial load. However, standardization of baseline conditions was aimed by using the same *Enterococcus faecalis* strain, identical inoculation volume, and the same incubation period for all samples.

The conventional colony-counting method was preferred for bacterial assessment. This method quantitatively detects only viable bacteria capable of growing in culture media and does not identify viable but non-culturable (VBNC) bacteria. This limitation may lead to an overestimation of antibacterial efficacy. In future studies, more comprehensive evaluation methods, such as confocal laser scanning microscopy (CLSM), may be preferred.

Although saline rewetting, H-file scraping, and multiple paper points were used to improve bacterial recovery, paper point-based sampling may still not recover all residual bacteria attached to the canal walls

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or dentinal tubules. Therefore, the reported CFU values should be interpreted within the limitations of this culture-based sampling method.

Another limitation of the present study is the use of a monospecific *E. faecalis* model with a 24-hour incubation period, which represents early-stage bacterial colonization rather than a mature, multispecies intraradicular infection. Therefore, the antibacterial efficacy of the tested agents may be overestimated compared with more complex clinical conditions. In addition, the 30-minute passive contact time was selected as a standardized laboratory screening duration and exceeds typical chairside irrigation times in pediatric endodontic treatment. Thus, future studies should evaluate these formulations using mature multispecies biofilm models and shorter, clinically applicable irrigation protocols.

## 5. CONCLUSION

The antibacterial activity observed for chitosan in this early-stage colonization model warrants further preclinical investigation; however, no conclusion regarding its clinical use as an irrigant can be drawn from the present findings. Papain-containing Brix 3000 showed lower antibacterial performance than sodium hypochlorite and chitosan under the present protocol. Given the simplified 24-hour monospecies model, absence of baseline bacterial quantification, and prolonged contact time, the findings cannot be extrapolated to mature biofilms or clinical conditions. Further studies using mature multispecies biofilm models and clinically relevant irrigation protocols are required.

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