



In vitro comparison of two antimicrobial pastes used in pediatric pulp treatment against *Enterococcus faecalis* ATCC 51299

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ABSTRACT

Introduction: Successful pulp treatment is achieved with proper disinfection of root canals, which is difficult to achieve completely in primary teeth due to the complex anatomy. Antimicrobial pastes can be an effective treatment alternative, reducing work time and material use. **Methods:** Reference strain *Enterococcus faecalis* ATCC 51299 was used. Bacterial suspensions adjusted to 0.5 McFarland standard were incubated for 24 h at 37°C with both pastes. Serial dilutions (10⁻¹-10⁻¹²) were done, and colony-forming units (CFU/ml) were determined. **Results:** Both pastes inhibited bacterial growth, with statistically significant differences (p<0.0001). The CTZ (LAB) paste demonstrated greater antimicrobial effectiveness compared to the CTZ(UNAM) paste and the Ultrapex® paste. **Conclusions:** CTZ and Ultrapex® pastes demonstrated antimicrobial activity against *E. faecalis*, with CTZ being more effective. CTZ represents a promising alternative for pulpal treatment in primary teeth.

Key words: CTZ paste; Ultrapex®; *Enterococcus faecalis*; antimicrobial activity; pediatric endodontics; primary teeth.

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RESUMEN

Introducción: El tratamiento pulpar exitoso se logra con una desinfección adecuada de los conductos radiculares, lo cual en dientes temporales es difícil de lograr en su totalidad debido a la compleja anatomía. Las pastas antimicrobianas pueden ser una alternativa de tratamiento eficaz, reduciendo tiempos de trabajo y material utilizado. **Objetivo:** Evaluar la actividad antimicrobiana “*in vitro*” de la pasta CTZ y Ultrapex® sobre *Enterococcus faecalis*. **Métodos:** Se utilizó la cepa de referencia *Enterococcus faecalis* ATCC 51299. Se prepararon suspensiones bacterianas ajustadas a la escala de 0.5 de McFarland. Las suspensiones fueron incubadas por 24 h a 37 °C en presencia de ambas pastas. Se realizaron diluciones seriadas (10⁻¹- 10⁻¹²) y se determinó el número de unidades formadoras de colonias (UFC/mL). **Resultados:** Ambas pastas mostraron inhibición del crecimiento bacteriano, con diferencias estadísticamente significativas ($p < 0.0001$). La pasta CTZ (LAB) presentó una mayor eficacia antimicrobiana en comparación con la pasta CTZ (UNAM) y la pasta Ultrapex®. **Conclusiones:** La pasta CTZ y la pasta Ultrapex® demostraron actividad antimicrobiana contra *E. faecalis*, siendo CTZ más efectiva. Su uso representa una alternativa viable en tratamientos pulpares de dientes temporales.

Palabras clave: pasta CTZ, ultrapex®; *Enterococcus faecalis*; actividad antimicrobiana; endodoncia pediátrica; dientes temporales.

INTRODUCTION

Pulpal disease in primary teeth represents a frequent clinical challenge in pediatric dentistry, largely due to the susceptibility of the dentin-pulp complex to injury. Dental caries is defined as a demineralization process of the tooth enamel, resulting from an ecological imbalance of the microorganisms present in the biofilm on the tooth surface. An increase in the prevalence of carious lesions has been observed in the pediatric population of Mexico, often leading to irreversible pulp involvement.^{1,2,3}

Microorganisms most frequently associated with pulp lesions in primary teeth are *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Streptococcus intermedius*, *Enterococcus faecalis*, *Clostridium* spp., and *Actinomyces israelii*, all of which are commonly implicated in irreversible pulpitis, pulp necrosis, and periapical abscesses.^{4,5,6} Within this group, the genus *Enterococcus* is of particular clinical relevance with *E. faecalis* being the most frequently isolated species and is strongly associated with endodontic treatment failure due to its high resistance to intracanal medications.^{7,8}

Factors explaining the high levels of resistance of *E. faecalis* includes the formation of dense and well-organized biofilms that hinder the penetration of antimicrobial agents, as well as its ability to invade and colonize dentinal tubules, where it remains protected from irrigation and environmental changes. Furthermore, *E. faecalis* can survive in nutrient-limited conditions and tolerate extreme pH variations, including the alkaline levels induced by calcium hydroxide, which enhances its persistence in endodontic failure.^{7,8}

Pulp treatment remains essential for preserving primary teeth, although complete disinfection is challenging due to their complex anatomy.^{4,9-11} Calcium hydroxide-iodoform pastes are the most widely used obturation materials in pediatric dentistry.¹² Commercial products such as Vitapex®, Metapex®, and Ultrapex® are considered ideal for filling primary canals.¹³ However, these materials can undergo rapid resorption —compromising apical sealing— along with progressive loss of radiopacity and limited antimicrobial efficacy against certain bacterial genera.^{11,12}

In Japan, a NO- instrumentation endodontic technique using antibiotic pastes —particularly CTZ, composed of chloramphenicol, tetracycline, and zinc oxide-eugenol— was developed for pulp disinfection.^{8,15} Although simple, low-cost, and broad-spectrum, it presents drawbacks such as crown discoloration, variable formulation quality and limited long-term evidence with recommendations to avoid its use in children under three years.¹⁵ Guedes et al., (2006) further demonstrated its antimicrobial potential, reporting that CTZ produced the largest inhibition halos against reference strains commonly associated with pulp necrosis, while Vitapex®, showed no activity.¹⁶

At the Dental Specialty Unit (DEFENSA), a considerable proportion of paediatric patients require emergency care due to pain and infection. Notwithstanding the ongoing efforts to prevent such complications, a significant proportion of patients present with irreversible or necrotic pulpal conditions. These cases frequently arise within stringent time constraints, rendering antimicrobial pastes a pragmatic therapeutic option as they curtail treatment time and instrumentation demands.



The utilisation of CTZ paste as an alternative in pulpectomy procedures for primary teeth has demonstrated favourable clinical outcomes. However, despite the documented antimicrobial properties of both CTZ and Ultrapex[®], the paucity of direct comparative in vitro evidence underscores the necessity for further investigation to ascertain their relative efficacies. The objective of the present study was to compare the in vitro antimicrobial activity of CTZ paste and Ultrapex[®] against *E. faecalis* ATCC 51299 to determine whether statistically significant differences exist between both formulations under standardised laboratory conditions.

Materials and Methods

An experimental, comparative in vitro study was conducted using the *E. faecalis* ATCC 51299 strain, which was provided by the Bacteriology Laboratory strain bank at the National School of Biological Sciences (IPN). For the microbiological assays, Müller-Hinton[®] (MH) medium was utilised for both agar plates and liquid media, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (2024).

Preparation of the bacterial strain

The reference strain was thawed and streaked on 5% blood agar using the cross-streak technique. Subsequently, the plates were subjected to an incubation process at a temperature of 37°C for a duration of 24 hours. The viability and purity of the sample were confirmed through conventional tests, including Gram staining, catalase, and oxidase, among others. Cryovials were prepared for the preservation of the strain at a temperature of -70°C.

Preparation of the pastes

The paste assays were performed using approximately 30 mg, based on the commercial dosage indicated by the Ultrapex[®] syringe packaging. The specified amount of solution was dispensed into each well of a 24-well microplate.

CTZ paste was procured from the National Autonomous University of Mexico (UNAM). For its preparation, 30 mg of CTZ powder (UNAM) was meticulously weighed on a glass slab, and two drops of eugenol (approximately 100 µL) were added with the objective of achieving a semi-solid, homogeneous, non-sticky paste. This consistency description is

applicable solely to the handling properties of the paste in clinical practice. In the present study, no root canals were inoculated; all evaluations were performed in vitro using 24-well microplates. Following the mixing process, the weight of each sample was once again verified using an analytical balance (Fisher Scientific[®]) prior to the inoculation of each well of the microplate.

Additionally, a CTZ (LAB) paste was prepared in the laboratory using each component based on the classical CTZ formulation in a 1:1:2 ratio (chloramphenicol, tetracycline, and zinc oxide). The paste was made by mixing 500 mg of generic tetracycline, 500 mg of chloramphenicol (PiSA[®]), 1000 mg of zinc oxide (Viarden[®]), and one drop of eugenol (approximately 50 µL). The components were then amalgamated on a glass slab using a metal spatula, continuing until a homogeneous, semi-solid, workable, and stable mixture was obtained, as previously described. Stock solutions were subsequently prepared at a concentration of 30 mg/mL.

Bacterial suspension and assay setup

The working bacterial suspension was adjusted in Müller-Hinton (MH) broth to 0.5 on the McFarland scale, corresponding to approximately 1.5×10^8 CFU/mL (CLSI, 2024). The three formulations previously described were evaluated: Ultrapex[®], CTZ (UNAM), and CTZ (LAB). In a 24-well microplate (Thermo Scientific[®]), the bacterial suspensions were distributed with each of the pastes (30 mg). The following controls were included: bacterial suspension without paste (growth control), culture medium alone (sterility control), and culture medium with paste (media interference control). This last control was included to assess whether the paste altered the culture medium conditions (e.g., or pH, turbidity, precipitation) that could lead to non-specific inhibition or artificial bacterial proliferation. No alterations were detected, thereby confirming that the paste did not interfere with the medium under the experimental conditions or that any contamination was not observed during the manipulation. In order to ensure the reliability of the results, three replicates of each bacterial suspension, in addition to controls, were performed for each paste. The microplates were then subjected to an incubation process in a New Brunswick Scientific[®] shaking incubator, operating at a speed of 150 revolutions per minute and a temperature of 37 degrees Celsius, for a duration of 24 hours. Following a 24-hour period, serial dilutions were performed to ascertain the concentration of colony-forming units per millilitre (CFU/mL).



Determination of CFU/mL

To determine the number of colony-forming units per millilitre (CFU/mL), serial dilutions were prepared from an aliquot of the bacterial suspension (Müller-Hinton) from each experimental well (containing bacteria and paste). Dilutions ranged from 10^{-1} to 10^{-12} .

For each experimental replicate, serial dilutions (10^{-10} , 10^{-11} , 10^{-12}), were plated in parallel on Müller-Hinton agar in triplicate. Plates were incubated at 37°C for 24 hours, and CFU were counted from the dilution that yielded 30-300 colonies, in accordance with standard microbiological quantification criteria.

Statistical analysis

Prior to conducting the analysis of variance (ANOVA), the normality of the data was evaluated using the Shapiro-Wilk

test. Subsequently, a multiple comparisons analysis was performed using Tukey's test, with the Epidat® statistical software employed for mean comparison. The objective of this study was to ascertain whether the variability observed in the arithmetic mean was attributable to random chance. A significance threshold of $p < 0.0001$ was established; therefore, values equal to or lower than this threshold were interpreted as statistically significant differences.

RESULTS

Bacterial Strain

Viability and purity of the strain was confirmed conventional tests, including Gram staining, catalase, and oxidase, among others (Figure 1). All the cryovials were prepared to preserve the strain at -70°C .

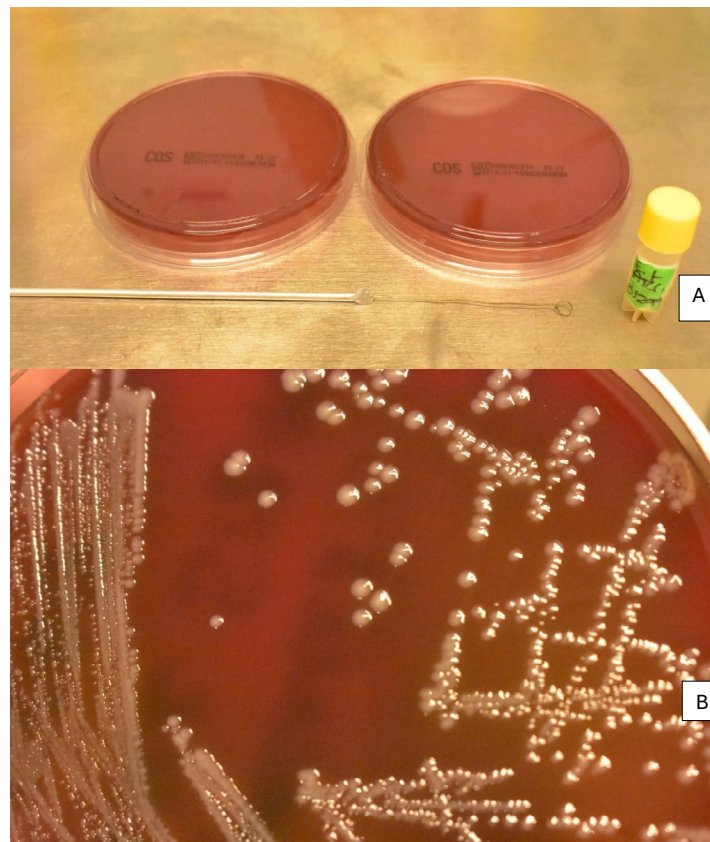


FIGURE 1. Viability and purity testing was performed on Casman agar (A). *Enterococcus faecalis* colonies are typically small, smooth, circular, and grayish white and non-hemolytic.



Preparation of the pastes

The CTZ (UNAM) paste was acquired from UNAM and it was prepared as previously described. The distribution of the assay in the 24-well microplate is observed in Figure 2.

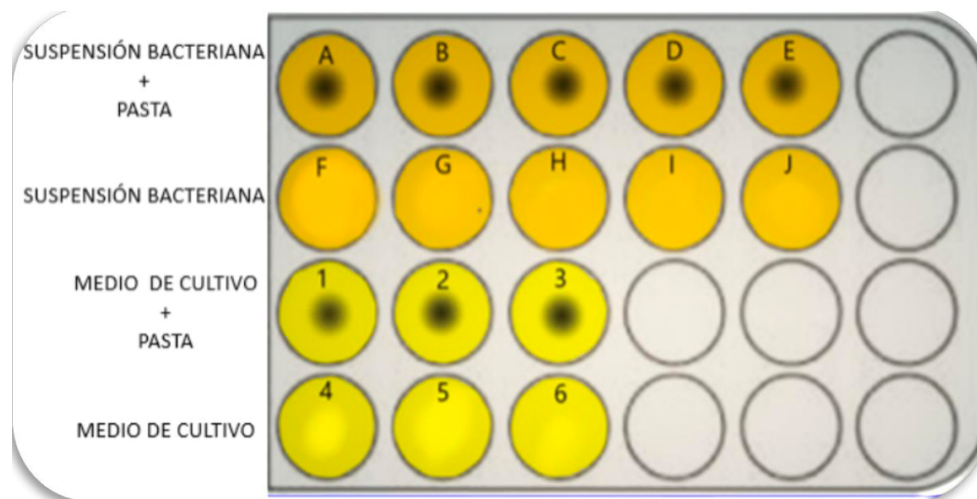


FIGURE 2. Distribution of the assays. Wells A-E contain bacterial suspension plus paste. Wells F-J contain bacterial suspension only. Control wells 1-3 contain culture medium plus paste. Control wells 4-6 contain culture medium only.

The initial assay performed with CTZ (UNAM) paste was not considered representative for analysis because the paste exhibited an oily separation that prevented homogeneous contact with the bacterial suspension (Figure 3). Due to the

oily separation observed in the initial CTZ (UNAM) paste preparation, which prevented homogeneous contact with the bacterial suspension, the initial results were excluded and a pre-treatment was done.

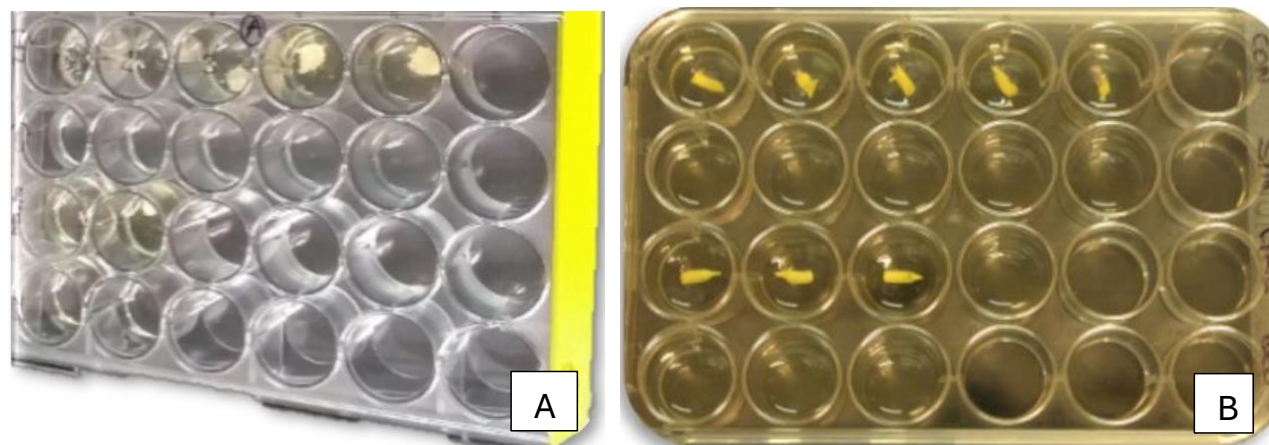


FIGURE 3. Distribution of the assays with (A) CTZ (UNAM) paste and (B) Ultrapex® paste. A noticeable separation can be observed in the CTZ (UNAM) paste, which prevents homogeneous dispersion throughout the well. In contrast, Ultrapex® exhibits uniform and consistent distribution in the bacterial suspension, allowing adequate contact between the paste and the culture medium.



To standardize the experimental conditions between formulations, both pastes were prepared as previously described, spread on a glass slab and dehydrated in an incubator at 38 °C for 24 hours (Figure 4). However, the CTZ (LAB) paste

was prepared directly from powdered components requiring only one drop of eugenol volume to achieve workable consistency, following the handling conditions recommended for this formulation.



FIGURE 4. CTZ(UNAM) paste mixture prior to the dehydration process.

The stock solutions were prepared by weighing 30 mg of the previously dehydrated powder using a Fisher Scientific A-250[®] analytical balance. The powders were then ground in an Omni[™] tissue homogenizer in Eppendorf tubes with 1 mL of sterile PiSA[®] water (Figure 5). Sterile water was used

following the CLSI 2024 guidelines for solvents and diluents in antimicrobial stock preparations for ensuring comparable consistency, solubility and direct contact conditions across all assays.¹⁷

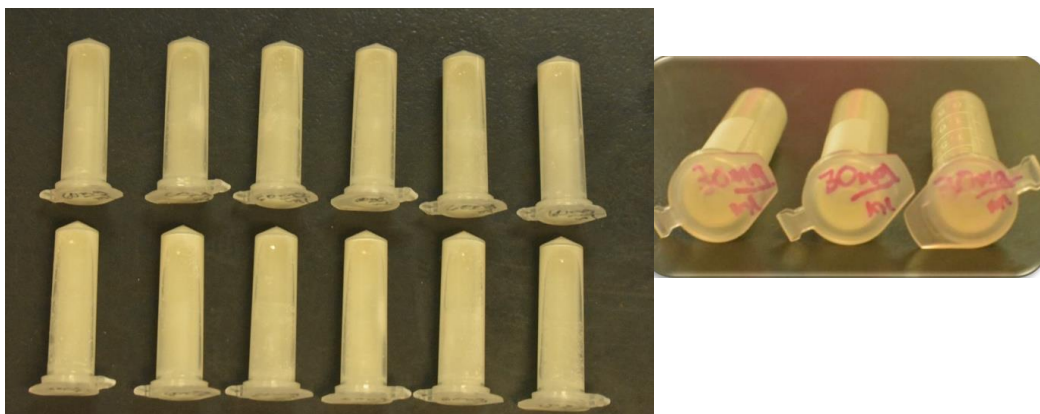


FIGURE 5. Stock solutions of 30 mg/mL of the CTZ paste.



Determination of CFU/mL

A second standardized assay was then performed. All reported results and statistical analyses in this study are based on this standardized phase (Figure 6). A dilution from

the stocks were done according to CLSI (2024), since 30 mg is the Minimum Inhibitory Concentration (MIC) for chloramphenicol and tetracycline. This CMI represents the lowest concentration of an antimicrobial drug that prevents the visible growth of a microorganism in a laboratory setting.

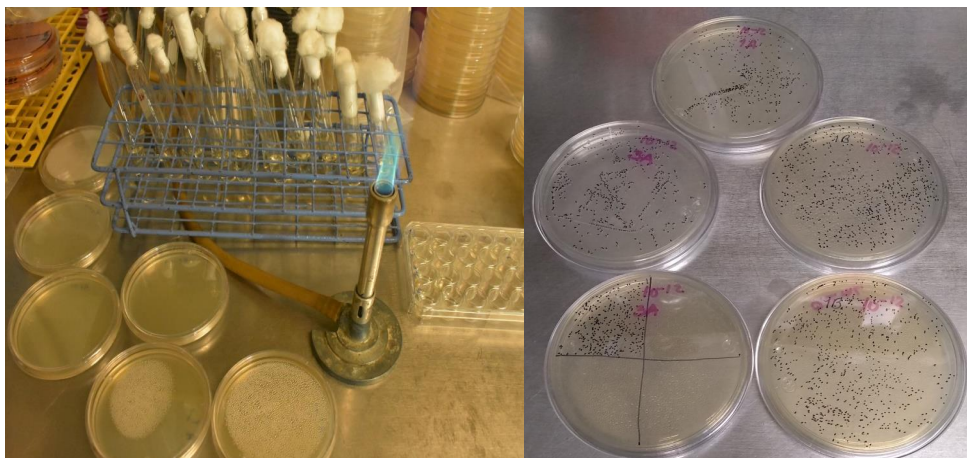


FIGURE 6. Material used for obtaining the CFU/mL. (A) Tubes used for serial dilutions and the corresponding MH agar plates are shown; (B) A representative image of the colonies obtained after the assay.

The colony counts obtained after the assay, compared to the control group, are shown in Table 1. The table presents the average values of colony-forming units per milliliter

(CFU/mL) from the three replicates performed for each paste. The control group maintained a stable microbial load of 5.4×10^{18} CFU/mL in all assays.

TABLE 1. CFU/mL counts for *E. faecalis* with the different pastes

| Paste | Assay 1 (avg) | Assay 2 (avg) | Assay 3 (avg) | Mean SD |
|--------------------------|-------------------------|-------------------------|-------------------------|--|
| | CFU/mL | CFU/mL | CFU/mL | CFU/mL |
| Growth control | 5.4 X 10 ¹⁸ | 5.4 X 10 ¹⁸ | 5.4 X 10 ¹⁸ | 5.4 × 10¹⁸ ± 0 |
| Ultrapex® | 4.20 X 10 ¹⁶ | 5.33 X 10 ¹⁶ | 6.95 X 10 ¹⁶ | 5.49 × 10¹⁶ ± 1.38 × 10¹⁶ |
| CTZ (UNAM) powder | 5.4 X 10 ¹⁸ | 4.96 X 10 ¹⁸ | 6.4 X 10 ¹⁸ | 5.59 × 10¹⁸ ± 7.38 × 10¹⁷ |
| CTZ (UNAM) paste | 5.4 x10 ¹⁸ | 5.5 x10 ¹⁸ | 5.6 x10 ¹⁸ | 5.50 × 10¹⁸ ± 1.00 × 10¹⁷ |
| CTZ (LAB) powder | 8.7 x10 ¹⁵ | 2.47 x10 ¹⁶ | 1.92 x10 ¹⁶ | 1.75 × 10¹⁶ ± 8.13 × 10¹⁵ |
| CTZ (LAB) paste | NG | NG | NG | NG |

*NG. No bacterial growth observed.



Based on the CFU/mL quantification, the growth control maintained a stable bacterial load of 5.4×10^{18} CFU/mL across all assays. Ultrapex® demonstrated a reduction of approximately two logarithmic units compared to the control, with a mean value of $5.49 \times 10^{16} \pm 1.38 \times 10^{16}$ CFU/mL.

When performing the statistical analysis comparing the control group with CTZ (UNAM), both in powder and paste form, the resulting p-value was > 0.9999 , indicating no statistically significant difference between them. Thus, neither

formulation showed antimicrobial activity against the ATCC strain of *E. faecalis*.

In contrast, comparisons between the control vs. Ultrapex® and control vs. CTZ (UNAM) powder yielded $p < 0.0001$, indicating statistically significant differences. Therefore, Ultrapex® and the non-commercial CTZ paste demonstrated antimicrobial activity, reducing the bacterial count by two logarithmic units (Figure 7).

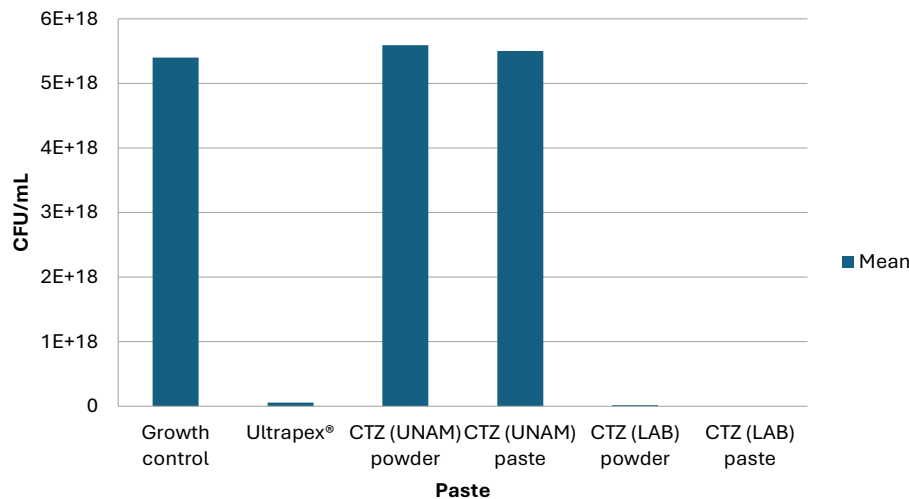


FIGURE 7. No significant differences were found between Control and CTZ (UNAM) powder or paste ($p > 0.9999$), confirming that neither CTZ (UNAM) formulation exhibited antimicrobial activity.

The bar graph indicates that the Growth control, CTZ (UNAM) powder, and CTZ (UNAM) paste demonstrated mean CFU/mL values around 10^{18} , with no discernible decrease in bacterial load. Conversely, Ultrapex® and CTZ (LAB) powder had significantly lower results, around 10^{16} CFU/mL, indicating a reduction of around two logarithmic units relative to the control. The CTZ (LAB) paste exhibited no observable bacterial growth, confirming total inhibition under the experimental circumstances.

The analysis of CFU/mL values revealed clear differences among the tested groups. The Control, CTZ (UNAM) powder, and CTZ (UNAM) paste groups showed bacterial counts within the 10^{18} range, with no statistically significant differences between them (Tukey, $p > 0.9999$) (Figure 8).

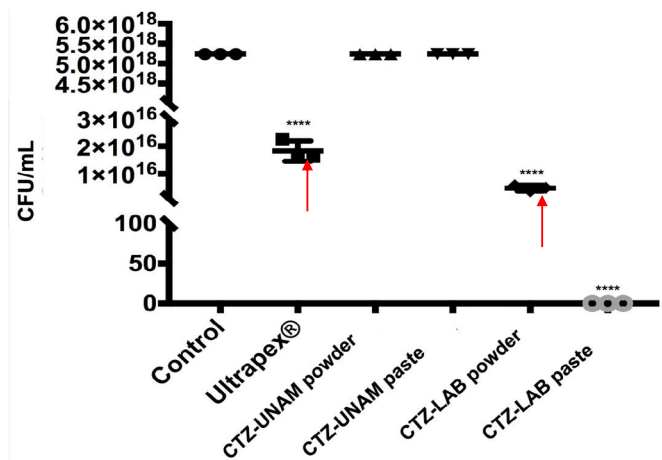


FIGURE 8. CFU/mL values of *E. faecalis* after exposure to the tested pastes (log scale). Control, CTZ (UNAM) powder, and CTZ (UNAM) paste showed comparable bacterial counts with no significant differences (Tukey, $p > 0.9999$).

Ultrapex® demonstrated a significant reduction in bacterial load to the 10^{16} range (Tukey, $p < 0.0001$), showing lower CFU/mL values compared with the Control and both CTZ (UNAM) formulations. Similarly, the CTZ (LAB) powder exhibited a significant decrease in CFU/mL (Tukey, $p < 0.0001$), while the CTZ (LAB) paste showed no detectable bacterial growth, differing significantly from all other groups (Tukey, $p < 0.0001$). Overall, these findings indicate statistically significant reductions for Ultrapex®, CTZ (LAB) powder, and CTZ (LAB) paste, whereas no reduction was observed for the CTZ (UNAM) formulations.

DISCUSSION

Diverse clinical studies have documented the efficacy of various materials employed in pulpal treatments of primary teeth. Nevertheless, the evidence from *in vitro* studies that consistently demonstrates the ability of these materials to inhibit bacterial growth remains limited.¹⁸

In the present study, the antimicrobial activity of the pastes was evaluated under *in vitro* conditions. An initial investigation was conducted to evaluate the antimicrobial properties of Ultrapex® paste. This investigation was conducted in accordance with the methodology established by Velasco (2012), in which filter paper disks were impregnated with Ultrapex®. In Velasco's study, the inhibition halos were described as minimal. In addition, no inhibition halos were observed in the laboratory, likely due to the specific properties of Ultrapex®'s composition, which does not diffuse well in agar-based culture media (data not shown).

CFU/mL counts were determined after the direct contact of the bacterial *E. faecalis* suspension with CTZ and Ultrapex® pastes. No *in vitro* studies were found evaluating the antimicrobial activity of Ultrapex® paste using CFU/mL counts; however, its clinical use has resulted in successful outcomes in eliminating signs and symptoms in patients.¹⁹

The present study reinforces the notion that the antimicrobial performance of endodontic pastes depends not only on their chemical composition but also on their formulation quality, handling characteristics, and interaction with the substrate. Our findings showed clear discrepancies between laboratory-standardized CTZ preparations and the commercially acquired compounded CTZ paste (CTZ UNAM), which is consistent with the variability reported in previous studies evaluating antibiotic-based formulations.^{15,16,18}

This study confirmed the *in vitro* activity of the two antimicrobial agents which are part of the CTZ paste formulation,

as well as the antimicrobial properties of zinc oxide and eugenol. CTZ paste is considered a compounding formula, meaning that there is no standardized control over the quality and source of its components, which may influence its effectiveness in inhibiting bacterial growth. This was evident since the CTZ (UNAM) formulation showed no antibacterial activity, even when the antibiotic content was used at or above the minimum inhibitory concentration (MIC) values described by CLSI. Bacterial growth was comparable to the untreated control group, with uncountable colony numbers, indicating that the acquired paste may not have had the expected composition or concentration of active antibiotics. This observation suggests the need for chemical analysis of the CTZ paste used in this study to verify its formulation and content.⁹

It is important to emphasize that the objective of the present study was to evaluate the *in vitro* antimicrobial effectiveness of commercially available and laboratory-prepared pastes against reference strain of *Enterococcus faecalis* rather than to assess their chemical composition. The CTZ(UNAM) paste was used as acquired, reflecting its actual clinical use. Therefore, although chemical characterization could provide additional information regarding formulation variability, it was beyond the scope of this study and does not compromise the interpretation of the antimicrobial results obtained.

Ultrapex® demonstrated a moderate but statistically significant reduction in *E. faecalis* counts, reducing bacterial load by approximately two logarithmic units. Although this effect was limited compared with CTZ (LAB), it aligns with the known mechanism of calcium hydroxide–iodoform pastes, which exert antimicrobial effects through hydroxyl ion release and sustained alkaline pH.¹⁹ However, *in vitro* models have shown limited efficacy of calcium hydroxide pastes against facultative anaerobes such as *E. faecalis*, explaining the incomplete inhibition observed in this study.

The performance of CTZ pastes differed markedly depending on the formulation. The CTZ (UNAM) paste—despite being widely used in clinical settings—did not reduce bacterial growth compared with the control. This result is supported by evidence indicating that compounded CTZ pastes can present substantial variability in antibiotic concentration, stability, and even degradation when stored under non-controlled conditions.^{18,19} The oily phase separation observed suggests inadequate homogeneity, which likely hindered the diffusion and direct contact of active compounds with bacterial cells. Similar inconsistencies have been described by Silva et al. (2019), who emphasized that CTZ variability significantly affects reproducibility in laboratory studies and possibly in clinical outcomes.



In contrast, the CTZ (LAB) formulation—standardized from raw antibiotics and freshly prepared—showed the greatest antimicrobial potency, including complete inhibition in the paste form. This finding is consistent with the classical evidence of Sato et al. (1992) and Takushige et al. (2004), both of whom demonstrated that when CTZ is precisely mixed in correct proportions, it produces strong antibacterial activity against both anaerobic and facultative microorganisms commonly present in necrotic primary teeth.

Our study further highlights the methodological importance of evaluating non-diffusible pastes using CFU/mL quantification rather than diffusion-based tests, as agar-based methods may falsely suggest lack of activity for materials with limited diffusion, such as Ultrapex®. The direct-contact approach employed in this study allowed a more accurate assessment of the actual antimicrobial effect of the pastes.

Clinically, the superior performance of CTZ (LAB) underscores the potential of CTZ when prepared under controlled, standardized conditions. This suggests that the inconsistent clinical outcomes reported for CTZ may be attributable to formulation variability rather than inherent limitations of the material. Standardization of CTZ preparation could therefore enhance its predictability and reliability in pediatric pulpectomy, particularly in cases where rapid bacterial reduction and minimal instrumentation are required. By contrast, the lack of antimicrobial effect observed in CTZ (UNAM) highlights the need for regulatory oversight of compounded dental materials to ensure therapeutic efficacy and patient safety.

Overall, the differences observed in this study emphasize that not all CTZ pastes behave similarly, and that formulation quality plays a decisive role in antimicrobial performance. Further studies using chemical characterization techniques (HPLC, mass spectrometry) are warranted to determine the stability and concentration of antibiotic components in commercial CTZ pastes.

CONCLUSION

Although CTZ paste was proposed over 50 years ago as an alternative pulpal treatment for primary teeth, *in vitro* studies evaluating its ability to inhibit bacteria associated with pulpal infections are limited. In the present study, Ultrapex® paste demonstrated significantly greater inhibition of bacterial growth than the control group. However, CTZ (LAB) paste, which was prepared in a laboratory, demonstrated greater antimicrobial efficacy against the *Enterococcus*

faecalis strain. This strain is considered to be clinically relevant in cases of pulpal infection. These results suggest that CTZ paste has clinical potential as an effective therapeutic alternative, offering shorter application times and favourable outcomes, provided the correct concentrations of each component are present in the paste. Adding eugenol to the CTZ mixture improved its antimicrobial activity, providing further evidence of its efficacy against *E. faecalis*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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