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BASIC RESEARCH:

Effect of Different Concentrations of Triple Antibiotic Paste Used in Root Canal Disinfection on the *In Vitro* Formation of *Enterococcus faecalis* Biofilms

Efecto de diferentes concentraciones de pasta triple antibiótica utilizada en la desinfección del conducto radicular sobre la formación *in vitro* de biopelículas de *Enterococcus faecalis*

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ABSTRACT: *Enterococcus faecalis* biofilms are a major cause of persistent endodontic infection and treatment failure. Triple antibiotic paste (TAP) is widely used, yet the optimal concentration that maximizes antibiofilm efficacy while minimizing cytotoxicity remains uncertain. This study aims to determine the minimum inhibitory concentration (MIC) of a modified TAP (ciprofloxacin–metronidazole–amoxicillin) against *E. faecalis* and to evaluate the effect of clinical and subinhibitory (sub-MIC) concentrations on *in vitro* biofilm formation. *E. faecalis* was cultured in brain heart infusion. MIC was determined by broth microdilution with resazurin as a viability indicator. Biofilm assays were performed in 96-well plates preconditioned for antibiotic adsorption. Standardized inocula were incubated 48 h (with medium renewal at 24 h). Biofilms were detached by sonication, serially diluted, drop-plated on BHI agar, and enumerated as CFU/mL. Percentage biofilm reduction was calculated relative to growth controls. Experiments were run in triplicate. The MIC of TAP against planktonic *E. faecalis* was 0.038 µg/mL. All concentrations tested reduced biofilm formation significantly versus MIC. Sub-MIC levels of 0.076 and 3.0 µg/mL produced similar reductions ($p > 0.05$). Higher sub-MIC concentrations—6.1, 24, and 49 µg/mL—achieved reductions approaching 100%, comparable to the clinical concentrations of 1000 and 2500 µg/mL ($p > 0.05$). Findings reveal a dose-dependent antibiofilm effect and highlight that some sub-MIC exposures can outperform the MIC for biofilm control. TAP exhibits a dose-dependent antibiofilm effect

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against *E. faecalis*, and concentrations below those commonly used clinically can markedly suppress biofilm formation *in vitro*. Sub-MIC levels equivalent to 1/1280, 1/640, and 1/160 of the MIC showed outstanding efficacy, suggesting the potential for lower-dose TAP protocols that preserve antibacterial activity while reducing adverse effects and antimicrobial resistance risk. Further studies must validate sub-MIC TAP regimens in multispecies biofilms and clinical settings.

KEYWORDS: *Enterococcus faecalis*; Biofilms; Anti-Bacterial Agents; Endodontics; Microorganisms; Root Canal Therapy.

RESUMEN: La pasta triple antibiótica (PTA) se emplea ampliamente como medicación intracanal, aunque persiste la incertidumbre acerca de la concentración óptima que maximiza la eficacia antibiofilm y minimiza la citotoxicidad. Este estudio tuvo como objetivo determinar la concentración mínima inhibitoria (CMI) de una PTA modificada compuesta por ciprofloxacina, metronidazol y amoxicilina frente a *Enterococcus. faecalis*, y evaluar el efecto de concentraciones clínicas y subinhibitorias (sub-CMI) sobre la formación de biopelículas *in vitro*. Las cepas de *E. faecalis* se cultivaron en medio de infusión cerebro-corazón (BHI). La CMI se estableció mediante el método de microdilución en caldo utilizando resazurina como indicador de viabilidad celular. Los ensayos de biopelícula se realizaron en placas de 96 pozos preacondicionadas para la adsorción del antibiótico. Los inóculos estandarizados se incubaron durante 48 horas, renovando el medio a las 24 horas. Posteriormente, las biopelículas se desprendieron por sonicación, se realizaron diluciones seriadas y se sembraron en agar BHI para la determinación de unidades formadoras de colonias (UFC/mL). La reducción de biopelícula se expresó como porcentaje respecto al control de crecimiento. La CMI de la PTA frente a *E. faecalis* planctónico fue de 0,038 μg/mL. Todas las concentraciones evaluadas redujeron significativamente la formación de biopelículas en comparación con la CMI. Los niveles sub-CMI de 0,076 y 3,0 μg/mL mostraron reducciones similares ($p > 0,05$), mientras que las concentraciones de 6,1, 24 y 49 μg/mL alcanzaron reducciones cercanas al 100%, comparables a las concentraciones clínicas de 1000 y 2500 μg/mL ($p > 0,05$). Los resultados demuestran un efecto antibiofilm dependiente de la dosis y sugieren que concentraciones sub-CMI pueden inhibir de manera efectiva la formación de biopelículas. Estos hallazgos respaldan el potencial de utilizar protocolos con dosis reducidas de PTA que mantengan la eficacia antibacteriana y disminuyan los efectos adversos y el riesgo de resistencia antimicrobiana.

PALABRAS CLAVE: *Enterococcus faecalis*; Biopelículas; Agentes antibacterianos; Endodoncia; Microorganismos; Tratamiento del conducto radicular.

INTRODUCTION

Dental caries is an infectious disease of multifactorial origin that leads to demineralization and progressive destruction of the hard tissues of the tooth (1). When not treated in a timely manner, carious lesions may progress to pulpal inflammation (reversible pulpitis), which, without treatment, evolves into irreversible pulpitis and subsequent pulp necrosis, with extension of inflammation and damage to the alveolar bone, resulting in apical periodontitis (2). Most of these cases are associated with polymicrobial infections involving aerobic and anaerobic bacteria, where the reduction of oxygen tension favors the predominance of strict anaerobes (3).

In this context, endodontic treatment emerges as a safe and effective alternative to preserve teeth affected by caries or trauma, especially when pulp vitality is compromised (4). However, the anatomical complexity of root canals, including accessory canals and dentinal tubules, hinders the complete elimination of microorganisms through mechanical instrumentation, making chemical irrigation indispensable (5). This challenge is further complicated by the formation of bacterial biofilms, complex microbial communities that confer resistance to antimicrobial agents (6). Among the most relevant pathogens, *Enterococcus faecalis* stands out as a facultative anaerobe frequently associated with persistent endodontic infections due to its ability to form resistant biofilms, penetrate dentinal tubules, and survive under extreme conditions (7,8).

In immature teeth with open apices, pulp involvement due to caries or trauma requires regenerative approaches such as revascularization, which promotes apical closure through the stimulation of dental papilla stem cells (9). This technique surpasses conventional apexification by allowing continuous root development, provided that disinfection is balanced with cell preservation (10,11). For this reason, the selection of intracanal medications is critical. One agent that has demonstrated efficacy in pulp regeneration procedures, biopulpectomies, resorptions, and perforation repair is calcium hydroxide ($Ca(OH)_2$). This compound stimulates dentin bridge formation and reparative dentinogenesis, and promotes pulp tissue healing. Additionally, it exerts a bactericidal effect through the generation of free radicals, protein denaturation, and bacterial DNA damage (12). Although it has been the reference medication for many years, its effectiveness may vary depending on the clinical application and severity of pulpal injury (13).

Nevertheless, triple antibiotic paste (TAP) has shown effectiveness in regenerative endodontic procedures in teeth with necrotic pulp and immature apices (14). TAP consists of ciprofloxacin, metronidazole, and minocycline, broad-spectrum antibiotics that inhibit the growth of polymicrobial communities in root canals prior to regeneration (15). However, minocycline has been shown to cause tooth discoloration; therefore, some studies suggest its replacement with clindamycin or amoxicillin (16). Intracanal medication must be selected carefully, considering its concentration, location, and duration of application in order to achieve the desired antimicrobial effect without damaging surrounding tissues (12). This precaution is especially relevant in teeth of pediatric patients with open apices, where it is crucial to avoid toxic concentrations that, while inhibiting bacterial growth, may also impair stem cells required for complete apical formation (11).

Currently, there is no consensus on the optimal TAP concentrations for managing endodontic infections, and in clinical practice, these concentrations are often elevated (17,18). For this reason, the American Association of Endodontists (AAE) recommends preparing the antibiotic mixture at low doses (1–5 mg/mL) [19] to maintain antibacterial efficacy while ensuring biocompatibility with stem cells (20).

Repeated or prolonged use of these medications may generate adverse effects, such as local toxicity, altered tissue responses, or bacterial resistance, which necessitates careful evaluation of risks and benefits prior to their application (21). In cases of infected canals with periapical complications, several studies suggest performing treatment in multiple sessions using intracanal medication between appointments to optimize disinfection (12). Although this strategy may enhance antimicrobial efficacy, it requires a careful balance between clinical effectiveness and biological safety. Therefore, the objective of this study was to determine the Minimum Inhibitory Concentration (MIC) of TAP against *E. faecalis* and to evaluate its effect, along with that of different subinhibitory (sub-MIC) and

clinical concentrations, on the *in vitro* formation of monospecies biofilms.

MATERIALS AND METHODS

GROWTH CONDITIONS AND PREPARATION OF BACTERIAL SUSPENSIONS

E. faecalis ATCC 29212 (Microbiologics, St. Cloud, MN, USA) was used. The strain was preserved at −20 °C in 20% glycerol (Sigma-Aldrich, Missouri, MO, USA) and reactivated on Brain Heart Infusion (BHI) agar (Difco Laboratories, Le Pont de Claix, France). To facilitate growth, cultures were incubated at 37 °C under microaerophilic conditions with 5% CO_2 for 18 h. After the incubation period, cell suspensions were prepared by transferring colonies into 10 mL of BHI broth (Merck Millipore, Burlington, MA, USA). Turbidity of the suspensions was measured using a turbidimeter (Velp Scientifica, Usmate Velate, Italy) until reaching a value of 90 ± 5 NTU (Nephelometric Turbidity Units), equivalent to approximately 1.5×10^8 CFU/mL (colony-forming units per milliliter). Finally, cell suspensions were diluted to achieve a final concentration of 1.5×10^6 CFU/mL.

PREPARATION OF THE TRIPLE ANTIBIOTIC PASTE (TAP) STOCK SOLUTION

TAP was prepared according to the protocol described by Mandal *et al.*, with slight modifications (1). Ciprofloxacin 500 mg tablets (La Santé, Bogotá, Colombia), metronidazole 500 mg (La Santé, Bogotá, Colombia), and amoxicillin 500 mg (La Santé, Bogotá, Colombia) were used. The purity of each tablet was determined by discounting the proportion of excipients. Tablets were ground in a sterile mortar into a fine powder and mixed in equal proportions (1:1:1) to form the base paste. A TAP stock solution of 10 mg/mL was prepared using 3% dimethyl sulfoxide (DMSO) as the vehicle.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF TAP AGAINST *E. FAECALIS*

To quantitatively measure the *in vitro* inhibitory effect of TAP on planktonic *E. faecalis* cells, the broth microdilution method was performed using resazurin as a viability indicator, following Clinical and Laboratory Standards Institute (CLSI) recommendations with slight modifications (2). Briefly, two-fold serial dilutions of TAP were prepared in 96-well microtiter plates (Costar, Corning Inc., NY, USA) starting from the 10 mg/mL stock solution. For this, 100 µL of the stock solution was added to the first three wells and mixed thoroughly by pipetting with 100 µL of BHI broth. Then, 100 µL of this diluted solution was transferred to the next three wells containing an equal volume of BHI broth, and the procedure was repeated to obtain concentrations ranging from 5000 µg/mL to 0.005 µg/mL.

Once dilutions were prepared, 10 µL of standardized *E. faecalis* suspension (1.5×10^6 CFU/mL) was inoculated into each well, resulting in a final concentration of 1.5×10^5 CFU/mL. Growth controls (100 µL BHI broth + 10 µL of *E. faecalis* suspension at 1.5×10^6 CFU/mL) and sterility controls (100 µL sterile BHI broth) were included. Plates were incubated at 37 °C under microaerophilic conditions (5% CO_2) for 24 h. Cell viability was assessed using the protocol described by Wardani *et al.* and Elshikh *et al.* (3,4), based on resazurin as a redox indicator. At the end of incubation, 30 µL of 0.015% resazurin solution was added to each well. To prevent degradation of resazurin by light, plates were covered with aluminum foil. Plates were then incubated at 37 °C for 2 h to observe color changes. A reduction of resazurin from blue to pink indicated bacterial metabolic activity. The MIC was defined as the lowest TAP concentration at which no color change occurred,

indicating inhibition of bacterial growth. All experiments were performed in triplicate.

EFFECT OF DIFFERENT TAP CONCENTRATIONS ON THE FORMATION OF *E. FAECALIS* MONOSPECIES BIOFILMS

To evaluate the *in vitro* effect of TAP on *E. faecalis* biofilm formation, eight concentrations were selected: two clinically used concentrations, five subinhibitory concentrations (sub-MIC), and the MIC (Table 1). The assay was conducted in 96-well microtiter plates, to which 200 µL of each TAP concentration was added. Plates were homogenized in an orbital shaker (Thermo Scientific, USA) for 3 h at 37 °C to allow antibiotic adsorption to well surfaces. Antibiotics were then carefully removed by pipetting, and wells were air-dried at room temperature inside a vertical laminar flow hood (BioBase, Qingdao, China).

Subsequently, 200 µL of *E. faecalis* suspension (1.5×10^7 CFU/mL) was added to the pretreated wells, and plates were incubated at 37 °C under microaerophilic conditions (5% CO_2) for 24 h. After this period, culture medium was renewed by removing supernatants and adding 200 µL of fresh BHI broth. Plates were reincubated under the same conditions for an additional 24 h, completing a total of 48 h of incubation to promote biofilm formation. Growth control consisted of 200 µL of *E. faecalis* suspension (1.5×10^7 CFU/mL), while sterility control consisted of 200 µL of BHI broth. All assays were performed in triplicate.

QUANTIFICATION OF *E. FAECALIS* BIOFILM REDUCTION

After 48 h of biofilm formation, supernatants were discarded and wells were washed twice with 100 µL of 0.9% saline solution (Corpaul, Medellín, Colombia) to remove non-adherent bacteria. Then, 150 µL of 0.9% saline was added to each well, and biofilms were sonicated in an ultrasonic processor (QSonica Q500, Newtown, CT, USA) at 50% amplitude with a 30 s pulse to detach adherent bacteria. Serial dilutions (10^{-1} to 10^{-5}) of the sonicated suspensions were prepared, and 10 µL of each dilution was inoculated on BHI agar using the drop plate method. Cultures were incubated at 37 °C under microaerophilic conditions (5% CO_2) for 48 h, and viable cells were enumerated as CFU/mL.

To assess the effect of different TAP concentrations on *E. faecalis* biofilm formation, the percentage of biofilm reduction (5) was calculated using the following equation:

$$\%\ \text{Biofilm Reduction} = \frac{(CFU_{cc} - CFU_{final})}{CFU_{cc}} \times 100$$

Where CFUcc represents the CFU of the *E. faecalis* biofilm in the growth control, and CFUfinal corresponds to the CFU obtained after treatment with different TAP concentrations.

STATISTICAL ANALYSIS

A descriptive analysis of the percentage reduction of *E. faecalis* biofilm obtained with different TAP concentrations was performed, including mean, standard deviation, minimum, and maximum. To compare the percentage of biofilm reduction among MIC, sub-MIC, and clinical TAP concentrations, one-way ANOVA with multiple comparisons was applied, assuming non-homogeneous variances (Levene's test), followed by the Games-Howell post hoc test. Normality was assessed using the Shapiro-Wilk test. Analyses were performed using IBM® SPSS 27, and a p-value < 0.05 was considered statistically significant.

RESULTS

In this study, the effect of different TAP concentrations on the formation of monospecies *E. faecalis* biofilms was evaluated. The MIC of TAP was determined to be 0.038 µg/mL, which represented the lowest concentration at which no resazurin color change was observed, indicating inhibition of bacterial growth (Figure 1).

After establishing the MIC of TAP against *E. faecalis*, five subinhibitory concentrations (sub-MIC) and two clinical concentrations were selected to assess their effect on monospecies biofilm formation (Table 1). All concentrations tested showed significantly higher percentages of biofilm reduction compared to the MIC (0.038 µg/mL). The sub-MIC concentrations of 0.076 µg/mL and 3.0 µg/mL did not exhibit statistically significant differences in biofilm reduction percentages (GH test $p > 0.05$). However, the higher sub-MIC concentrations (6.1 µg/mL, 24 µg/mL, and 49 µg/mL) produced a notable decrease in biofilm formation, showing reduction rates close to 100%. These results demonstrated an effect comparable to that of the clinical concentrations of 1000 µg/mL and 2500 µg/mL (GH test $p > 0.05$) (Table 2).

Figure 2 illustrates the effect of the different TAP concentrations on reducing *E. faecalis* biofilm formation, indicating a direct relationship between TAP concentration and antibiofilm activity, suggesting a dose-dependent effect. Although the MIC was the lowest TAP concentration that inhibited visible growth of planktonic *E. faecalis* cells, its efficacy against biofilm-associated cells was limited compared to sub-MIC concentrations, confirming the inherent resistance of this species when growing in biofilm state.

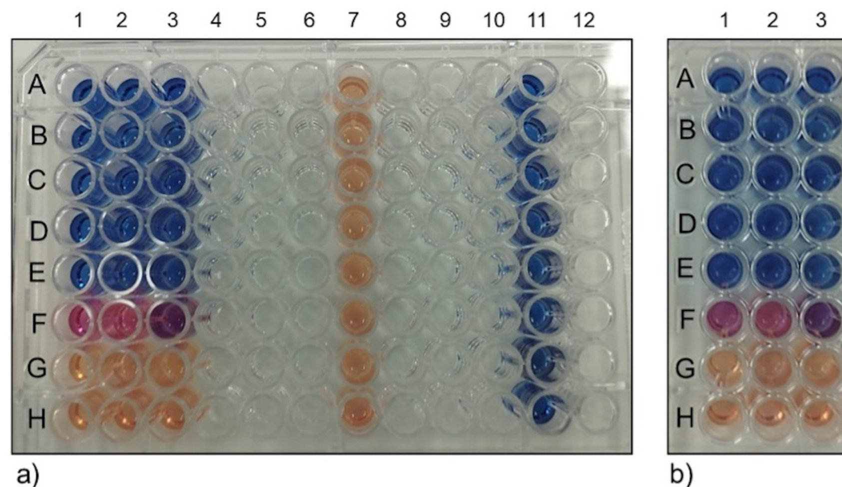

Figure 1. Determination of the MIC of TAP against *E. faecalis* ATCC 29212.
a) Rows A–H, columns 1, 2, and 3 correspond to a range of two-fold serial dilutions of TAP from 0.6 µg/mL to 0.005 µg/mL. Column 7 corresponds to the growth control, which shows a color change from the natural blue of resazurin to the reduced pink form. Column 11 corresponds to the sterility control. b) Row E shows no color change; therefore, the TAP concentration in these wells was taken as the MIC value (0.038 µg/mL).

Table 1. TAP concentrations evaluated in the *in vitro* formation of monospecies *E. faecalis* biofilm.

TAP concentrations	
2500 μg/mL	
1000 μg/mL	
sub-MIC concentrations	
49 μg/mL	1/1280 MIC
24 μg/mL	1/640 MIC
6.1 μg/mL	1/160 MIC
3.0 μg/mL	1/80 MIC
0.076 μg/mL	1/2 MIC
MIC	
0.038 μg/mL	

TAP:Triple antibiotic; sub-MIC: subinhibitory concentrations; MIC: Minimum Inhibitory Concentration.

Table 2. Percentage reduction of *E. faecalis* biofilms in the presence of different TAP concentrations.

		% Biofilm reduction		
TAP concentrations		**Mean ± SD**	**Minimum - maximum**	**p - Value**
Clinical concentration	2500 μg/mL[a]	100 ± 0.0	100 - 100	
	1000 μg/mL[a]	100 ± 0.0	100 - 100	
	49 μg/mL (1/1280 MIC)[a]	99.96 ± 0.05	99.91 - 100	<0.001
	24 μg/mL (1/640 MIC)[a]	97.35 ± 1.35	95.79 - 98.18	
Sub-MIC	6.1 μg/mL (1/160 MIC)[a,b]	96.03 ± 2.61	93.02 - 97.72	
	3.0 μg/mL (1/80 MIC)[a,b]	94.26 ± 1.62	93.03 - 96.09	
	0.076 μg/mL (1/2 MIC)[b]	89.77 ± 0.81	89.05 - 90.65	
MIC	0.038 μg/mL[c]	66.71 ± 2.52	64.42 - 69.30	

TAP:Triple antibiotic; sub-MIC: subinhibitory concentrations; MIC: Minimum Inhibitory Concentration; % Biofilm reduction: percentage of biofilm reduction; SD: standard deviation. One-way ANOVA; a,b,c: Games–Howell multiple comparisons. Concentrations with different letters indicate statistically significant differences in biofilm reduction ($p < 0.05$).

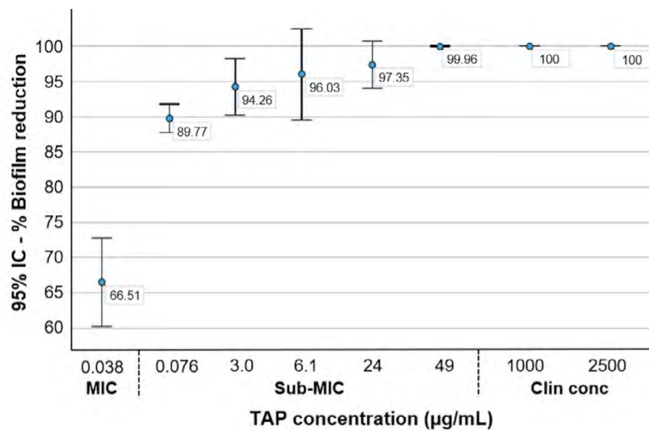

Figure 2. Effect of different TAP concentrations on the reduction of *E. faecalis* biofilm formation.
MIC: Minimum Inhibitory Concentration; sub-MIC: subinhibitory concentrations; Clin conc: clinical concentration.

DISCUSSION

The results of this study strengthen the available evidence on the efficacy of TAP in achieving a significant reduction of *E. faecalis* biofilms, even when concentrations considerably lower than those traditionally used in clinical practice are employed. These findings are consistent with previous research demonstrating that TAP, even at low concentrations, retains potent antibiofilm activity due to the synergistic action of its components against resistant bacteria (22,23). This aspect is particularly relevant since *E. faecalis* is recognized as one of the main agents responsible for endodontic failure, owing to its ability to survive under adverse conditions and its pronounced capacity to form highly resistant biofilms (3,7).

Our findings, which show that subinhibitory (sub-MIC) concentrations of TAP, such as 49 μg/mL (1/1280 MIC), can reduce biofilm formation by more than 99%, align with recent investigations reporting the strong antimicrobial potency of TAP even at low doses (20,24). This outcome is clinically relevant as it opens the possibility of optimizing the balance between antibacterial efficacy and biological safety, particularly in revascularization and regenerative endodontic procedures, where preservation of dental pulp stem cells is critical (9).

The rational and reduced use of antibiotics in endodontics not only limits adverse effects on tissues but also addresses a global concern regarding the increasing bacterial resistance arising from indiscriminate antimicrobial use. Guidelines from the World Health Organization and scientific societies emphasize the need to use the lowest effective dose possible to limit the emergence of resistant strains (WHO, 2023), in line with the results presented here. In our study, the efficacy of sub-MIC concentrations (6.1 μg/mL, 24 μg/mL, and 49 μg/mL) in reducing *E. faecalis* biofilm formation is highlighted. Clinically, the application of subinhibitory TAP concentrations could allow better preservation of pulp tissue vitality and regenerative potential, minimizing cytotoxicity without compromising antimicrobial activity (15). Nevertheless, it is important to emphasize that repeated exposure to sub-MIC concentrations may, under certain circumstances, promote the development of more resistant biofilms or induce bacterial resistance mechanisms; thus, caution and careful clinical supervision are recommended (24).

Furthermore, this study highlights a fundamental difference between the behavior of *E. faecalis* in planktonic state compared with its organization in biofilms. While the minimum inhibitory concentration (MIC) is sufficient to inhibit the growth of free-living bacteria, it is not always the most effective against mature biofilms. This higher resistance is attributed to the extracellular matrix, variability in gene expression, and the presence of persister cells that are less susceptible to antibiotic action (6,25).

Several *in vitro* studies have evaluated the effectiveness of TAP against *E. faecalis* in different experimental models, reporting significant reductions in bacterial viability and a dose-dependent bactericidal effect, both in biofilms and in penetration into dentinal tubules and infected dentin. The clinical use of TAP has been recommended by the American Association of Endodontists as one of the most widely used intracanal therapies in regenerative endodontic procedures due to its broad antimicrobial spectrum (19). However, the risk of tooth discoloration associated with minocycline, one of the components of the original formulation, has prompted the search for alternatives that preserve clinical effectiveness without compromising the patient's esthetic outcome (26). In this context, the present study evaluated a modified formulation in which minocycline was replaced by amoxicillin, and this combination retained significant efficacy in reducing *in vitro E. faecalis* biofilms, suggesting its potential as an alternative in the triple antibiotic mixture.

Although this is an *in vitro* study, our results support the efficacy of TAP at low concentrations in inhibiting the growth and reducing the biofilm formation of *E. faecalis*. However, the main limitation of this study is that the evaluation was performed on monospecies biofilms, which do not accurately reflect the complexity of intraradicular infections. In the oral environment, these infections are typically polymicrobial and influenced by interspecies interactions that may alter microbial susceptibility to antibiotics (27) and, consequently, the clinical effectiveness of TAP. Therefore, further studies involving multispecies biofilms that better simulate clinical conditions are required.

CONCLUSION

The results of this study demonstrate that the antibiofilm effect of TAP against *E. faecalis* is dose-dependent. It was shown that concentrations lower than those routinely used in clinical practice can significantly inhibit *in vitro* biofilm formation. In particular, sub-MIC concentrations equivalent to 1/1280, 1/640, and 1/160 of the MIC exhibited remarkable antibacterial efficacy. These findings suggest a relevant clinical impact by enabling the use of lower TAP doses without compromising antibacterial activity, which could help minimize adverse effects and limit the development of bacterial resistance mechanisms to antibiotics. Further studies are required to support the clinical use of sub-MIC TAP concentrations in controlling intraradicular E. faecalis biofilms.

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FUNDING: This research did not receive grants.

DECLARED NONE: This *in vitro* study does not require bioethics committee approval.

DATA AVAILABILITY STATEMENT: The datasets used and/or analyzed during the current study are available from the corresponding author.

AUTHOR CONTRIBUTION STATEMENT: Conceptualization: E.P.V., C.M.B.C. and C. M.A.; Methodology: E.P.V., C.M.B.C. and C. M.A.; Formal analysis: E.P.V. and C.M.B.C.; Investigation: E.P.V., C.M.B.C. and C. M.A.; Data curation: E.P.V., C.M.B.C. and C. M.A.; Writing-original draft preparation: E.P.V., C.M.B.C. and C. M.A.; Writing-review and editing: E.P.V., C.M.B.C. and C. M.A.; Administration: EE.P.V. and C.M.B.C. All authors have read and agreed to the published version of the manuscript.

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