

***In vitro* evaluation of the susceptibility level of *Enterococcus faecalis* to intracanal medications**

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Abstract

The main objective of this study was to evaluate the susceptibility of *Enterococcus faecalis* to intracanal medications. To achieve this, a comparative *in vitro* study was conducted at the Universidad de Los Hemisferios' microbiology laboratory, using *Enterococcus faecalis* strains in contact with conventional tri-antibiotic pastes, modified tri-antibiotic pastes, and bi-antibiotic pastes to observe the presence of inhibition zones, which were subsequently measured with a digital vernier caliper. The bacteria were cultured in sterile Petri dishes containing bile esculin agar and incubated at 37.5 °C for 24 hours under aerobic conditions. After this period, the microorganism was inoculated into a test tube with physiological serum at a turbidity of 0.5 McFarland. Then, 100 µL of the solution was extracted and deposited in a Petri dish containing the same agar, and microorganisms were spread using a Digralsky loop. This procedure was repeated 20 times. The pastes were prepared for study by removing the coatings from the tablets of the medications, crushing them in a sterile mortar, and storing them in sterile, amber-colored containers for each drug for 14 and 28 days. The tri-antibiotic pastes (conventional and modified) were mixed in a 1:1:1 ratio, while the bi-antibiotic paste was mixed in a 1:1 ratio. Subsequently, all pastes were mixed with glycerin (vehicle) in a 1:1 proportion. Wells corresponding to each paste (100 µL) were opened in the previously inoculated Petri dishes, then incubated at 37.5 °C for 24 hours. After incubation, inhibition zones were measured using a digital vernier caliper. These measurements were recorded in Excel and statistically analyzed using the ANOVA test and HSD de Tukey. The same procedure was repeated at 14 and 28 days with the previously stored medications. The results showed that the modified tri-antibiotic paste exhibited inhibition zones greater than 27 mm, while the conventional tri-antibiotic and bi-antibiotic pastes presented inhibition zones greater than 21 mm. In conclusion, the study demonstrated that the *Enterococcus faecalis* strain is highly sensitive to the studied pastes, and storage time did not affect the effectiveness of any of the medications.

Keywords: *Enterococcus faecalis*, antibacterial, root canal medicaments

Introduction

One of the main objectives of endodontics is the removal of residual pulp tissue from the pulp chamber and root canals to enable optimal instrumentation, disinfection, and cleaning of the canals (Leiva-Vulasich, 2022) ^[1]. This process allows for a three-dimensional sealing of the canals, preventing voids or leakage (Kali, 2022), through the use of various commercially available materials such as gutta-percha cones and different endodontic sealers, which are essential for achieving an ideal apical sealed without surpassing the apical constriction (Li, 2021) ^[3].

The persistence of infection may be due to the formation of microbial biofilms – dynamic communities of interacting cells attached to solid substrates (Prada *et al.*, 2019) ^[4]– which are located in anatomically complex areas such as isthmuses, lateral canals, and dentinal tubules, complicating the cleaning and disinfection of the canal system (Versiani *et al.*, 2023) ^[5]. In this regard, one of the main factors associated with endodontic treatment failure is the persistence of microbial infection within the root canal system, with microorganisms surviving despite chemical-mechanical preparation (Prada *et al.*, 2019) ^[4].

Endodontic therapy also aims to eliminate or reduce the bacterial load within the root canals (Siqueira, 2022) ^[6], which is responsible for endodontic origin and is characterized by the presence of various microorganisms, including *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, *Candida albicans*, and *Enterococcus faecalis*. The latter is frequently found in root canal treatments that, for several reasons, have not had in successful outcome

(Algamdhi, 2020), and it exhibits virulence factors that enhance its adhesion to canal walls (Francisco *et al.*, 2020). *Enterococcus faecalis* is a bacterial species commonly found in secondary or persistent endodontic injuries. It is gram-positive and facultatively anaerobic (Bouillaguet *et al.*, 2018) ^[9], capable of surviving under adverse environmental conditions and during prolonged periods of nutrient deprivation. Moreover, it shows resistance to certain intracanal medications used during treatment (Ali *et al.*, 2020) ^[10, 11]. Through pili associated with biofilm formation, it can adhere to the external surface of the root and form dense biofilms (Momenijavid *et al.*, 2022) ^[12]. Therefore, the objective of this study is to evaluate the susceptibility of *Enterococcus faecalis* to intracanal medications based on their storage duration.

Materials and Methods

This study was designed as an experimental, comparative, *in vitro* investigation and was submitted for approval to the ethics committee of Universidad de Los Hemisferios. The research was conducted under sterile conditions in the university's microbiology laboratory. *Enterococcus faecalis* strains were used to evaluate the antimicrobial efficacy of conventional triple antibiotic paste, modified triple antibiotic paste, and bi-antibiotic paste. Susceptibility to this formulation was assessed by measuring inhibition zones and evaluating the reduction or elimination of bacterial biofilm.

Materials

- Petri dishes (20 units)
- 40 ml beakers
- Sterile stainless-steel cotton forceps

- Micropipettes (20 µl and 100 µl)
- Digiralsky spreader
- Inoculation loop
- Bunsen burners
- Test tube racks
- Electronic balance
- Digital vernier caliper
- Sterile amber containers (3 units)
- Laminar flow hood
- Incubator

Media Used

- bile esculin agar
- physiological saline solution

	Solutions	Test number
Positive Control Group	Conventional triple-antibiotic paste (metronidazole and ciprofloxacin and minocycline)	22
Study Group	Bi-antibiotic paste (metronidazole and ciprofloxacin)	22
	Modified triple-antibiotic paste (metronidazole and ciprofloxacin and amoxicillin)	22
Negative Control Group	Physiological saline solution	22

Bacterial Processing

Under aerobic conditions, bacteria were inoculated into a bile esculin agar Petri dish (initial plate) and incubated for 24 hours at 35.7 °C to allow strain development. After reactivation, three bacterial colonies from the original plate were collected using an inoculation loop and transferred to a test tube containing physiological saline.

For inoculation, 100 µl of the resulting solution was extracted and Turbidity was assessed every 5 minutes over 15-minute periods until 0.5 on the McFarland scale.

Deposited into a bile esculin agar Petri dish. The inoculation was performed using a Digiralsky loop. This sequence was repeated 22 times for each test solution.

Preparation of Tri antibiotic and Bi antibiotic Pastes

First, the coatings of the medication tablets were removed, and the tablets were ground in sterile mortars.

Second, powdered samples were placed in sterile amber glass jars, properly labeled with the corresponding drug name.

Glycerin was used as the vehicle (mixed in 1:1 ratio). The medicated pastes were prepared in the following formulations:

- **Paste A:** Conventional triple-antibiotic paste (metronidazole and ciprofloxacin and minocycline)
- **Paste B:** Bi-antibiotic paste (metronidazole and ciprofloxacin)
- **Paste C:** Modified triple-antibiotic paste (metronidazole and ciprofloxacin and amoxicillin)

Each antibiotic powder was used at 1mg in proportions of 1:1 (Bi-antibiotic paste) and 1:1:1 (tri-antibiotic paste), mixed with glycerin (vehicle) in a 1:1 ratio, forming a paste-like preparation of 1 mg/ml powder-to-liquid. This stage was carried out using a calibrated precision balance (Ohaus®, accuracy of 0.001g).

Sample

- *Enterococcus faecalis* strain ATCC 29212

Test Solutions

- Bi-antibiotic paste (metronidazole and ciprofloxacin)
- Modified triple antibiotic paste (metronidazole and ciprofloxacin and amoxicillin)

Negative Control Group

- Physiological saline solution

Positive Control Group

- Conventional triple antibiotic paste (metronidazole and ciprofloxacin and minocycline)

Interaction Between Medicated Pastes and *Enterococcus Faecalis*

Evaluations were conducted on three specific days: day 0, day 14 and day 28. On day 0, the pastes were prepared using freshly obtained components from tablets or capsules. On days 14 and 28, stored medications from the perspective time intervals were used. A total of 22 Petri dishes were used throughout the study.

Each dish contained two wells (15 mm apart) for placement of the test solutions. A representative portion of each paste was selected and divided into equal sub-portions of 100 µl using an electronic balance. For the negative control wells, 20 µl of physiological saline was deposited.

Finally, the plates were incubated at 35°C for 24 hours. Antimicrobial activity of the formulation was evaluated by measuring inhibition halos using a calibrated vernier caliper (Truper®, accuracy ±0.0005"/0.01mm), and results were compared using the Duraffourd scale (Checalla *et al.*, 2021)^[13], which defines the following parameters:

- **None:** ≤ 8 mm (-)
- **Sensitive:** 9-14 mm (+)
- **Very sensitive:** 15-19 mm (++)
- **highly sensitive:** ≥ 20 mm (+++)

Statistical Analysis

Data processing was performed using SPSS Statics version 25.0 (IBM Corp., Armonk, NY, USA). To assess differences between groups, ANOVA was applied. When ANOVA indicated statistically significant differences ($p < 0.05$), Turkey's HSD post hoc test was used for multiple comparisons to determine which groups differed from each other.

Results

In the present study, a total of 88 wells were evaluated, of which 22 were due to the absence of inhibition halos; these correspond to the wells in the negative control group, which lacks antimicrobial activity. At 24 hours (day 0), 6 plates were measured; at 14 day, 7 plates were measured; and at 28 days, 9 plates were measured. These data were recorded in an Excel spreadsheet, specifying the day and plate number for each paste evaluated.

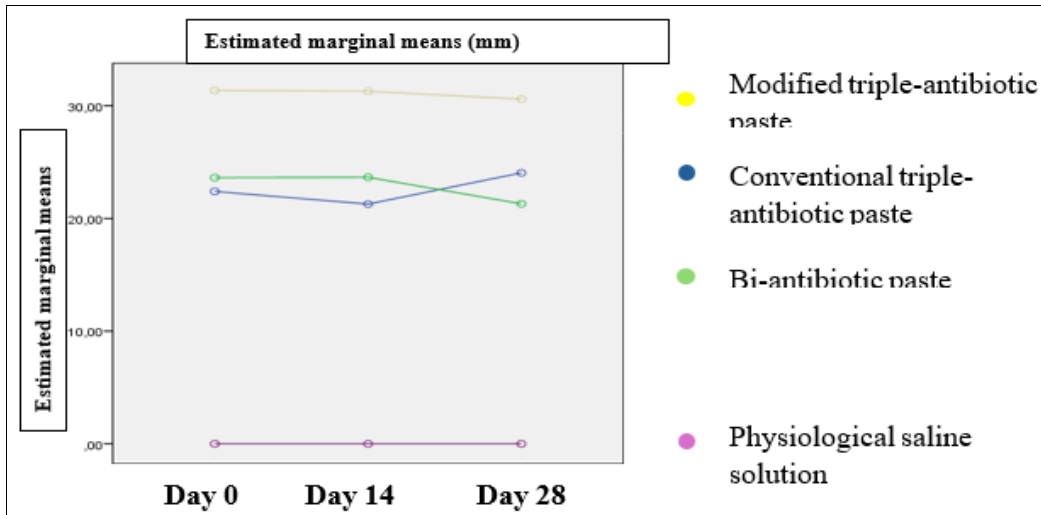


Fig 1: Relación fecha/ halo de inhibición. Línea amarilla: pasta triantibiótica modificada. Línea verde: pasta biantibiótica. Línea azul: pasta triantibiótica convencional.

Group C (modified triple-antibiotic paste) exhibited higher and more consistent values, with inhibition halos whose diameters were significantly larger compared to the other evaluated pastes. The double antibiotic paste showed a progressive decline in activity on days 14 and 28,

suggesting a loss of antimicrobial efficacy. The conventional triple antibiotic paste began with moderate halos, showed a slight decrease at day 15, and demonstrated increased antibiotic activity by day 28.

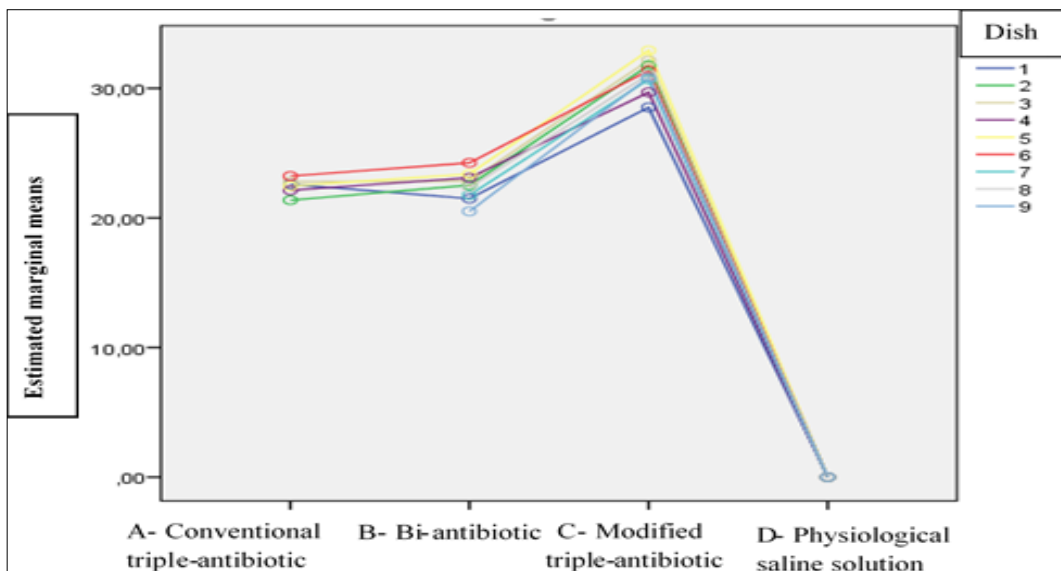


Fig 2: Tamaño de los halos de inhibición en las nueve placas de estudio a los 28 días

Figure 2 shows the size of the inhibition halos in the nine study plates at 28 days. It can be observed that the three antimicrobial formulations reached high and comparable values; however, the modified triple antibiotic paste

exhibited a larger inhibition halo diameter, suggesting superior antimicrobial activity compared to the other preparations.

Table 1: Tukey’s HSD Post hoc Test

Inhibition Halo	N	Subset		
		1	2	3
D- Physiological saline solution (-)	22	0		
A- Conventional triple-antibiotic paste (+)	22		22,4675	
B- Bi-antibiotic	22		22,6882	
C- Modified triple-antibiotic paste	22			31,025
Sig.		1	0,981	1

After detecting significant differences ($p < 0,05$) through analysis of variance (ANOVA), Tukey’s HSD post hoc test was applied, grouping the four treatments into three subsets.

It was observed that the conventional paste and the bi-antibiotic paste, both located in subset 2, exhibited similar and moderate antimicrobial activity (22.5 mm), classified as

highly sensitive (+) according to the Durafourd scale. Likewise, the modified triple-antibiotic paste, located in subset 3, achieved a mean diameter of 31,025 mm, also

classified as highly sensitive (+) by the same scale, representing a significantly higher value than other treatments and suggesting it to be the most effective option.

Table 2: Hypothesis testing. Values marked with * indicate statistically significant differences.

(I)Halo of Inhibition		Difference of measurements (I-J)	Error t _{íp.}	Value-P
A- T Conventional triple-antibiotic paste (+)	B- Bi-antibiotic	-,2207	,66186	,987
	C- Modified triple-antibiotic paste	-8,5575*	,66186	,000
	D- Physiological saline solution (-)	22,4675*	,66186	,000
B- Bi-antibiotic	A- Conventional triple-antibiotic paste (+)	,2207	,66186	,987
	C- Modified triple-antibiotic paste	-8,3368*	,48336	,000
	D- Physiological saline solution (-)	22,6882*	,48336	,000
C- Modified triple-antibiotic paste	A- Conventional triple-antibiotic paste (+)	8,5575*	,66186	,000
	B- Bi-antibiotic	8,3368*	,48336	,000
	D- Physiological saline solution (-)	31,0250*	,48336	,000
D-Physiological saline solution (-)	A- Conventional triple-antibiotic paste (+)	-22,4675*	,66186	,000
	B- Bi-antibiotic	-22,6882*	,48336	,000
	C- Modified triple-antibiotic paste	-31,0250*	,48336	,000

Figure 3 shows the results of the hypothesis test, indicating the p-value corresponding to each comparison. Negative meaning difference values (-) suggest that the second paste was more effective than the first, whereas positive values (+) indicate greater effectiveness of the first paste. Accordingly, when comparing the conventional tri-antibiotic paste (A) with the modified tri-antibiotic paste (C), it is determined that the modified formulation exhibits a significantly greater effect than the conventional formulation.

Discussion

The results obtained in the present study demonstrate that the bi-antibiotic paste exhibited a progressive decline in its antimicrobial efficacy on days 14 and 28. This reduction may be associated with the loss of stability of its active ingredients or with the absence of a third antibiotic agent to enhance its spectrum of action. This pattern was confirmed by Montero *et al.* (2018) [16], who reported that formulations containing fewer antibiotic agents showed a notable decrease in antimicrobial potency, thereby compromising their therapeutic capacity.

Regarding the conventional tri-antibiotic paste, a variable response was observed over time: it initially produced moderate inhibition zones, followed by a slight decrease in possibility of a later stage. Indeed, similar findings were reported by Wassel *et al.* (2022), who found that certain vehicles allow for sustained release of antibiotic combinations within the root canal system, potentially generating peaks of antimicrobial activity after application, attributed to the vehicle’s composition.

Statistical analysis using ANOVA revealed significant differences between groups (p<0.05), and the Tukey HSD post hoc test allowed the treatments to be grouped into three subsets. In this context, the modified tri-antibiotic paste was placed in an independent subset, with a mean inhibition halo diameter of 31,025 mm- a statistically superior value compared to the other formulations. These findings support its potential as the most effective alternative in terms of antimicrobial activity.

The results support the hypothesis that strategically integrating additional components into intracanal medicaments can enhance their therapeutic efficacy. This is further corroborated by Ulloa *et al.* (2024) [18], who

demonstrated that the tri-antibiotic paste exhibits efficient antimicrobial activity even in anatomically complex regions of the root canal system, attributed to the pharmacological complementarity of its components and their penetration capacity. As reported by Valan *et al.* (2021), the modified tri-antibiotic paste showed antimicrobial activity, enable the elimination of *Enterococcus faecalis* even at reduced concentrations.

Additionally, the direct comparison between the conventional tri-antibiotic paste (A) and the modified version (C) revealed a significantly greater antimicrobial effect in the latter. This suggests that modifications to the formulation may be considered a viable alternative in complex clinical scenarios, particularly in the treatment of persistent endodontic infections. In this regard, Parashar *et al.* (2020) [20] reported that substituting minocycline with clindamycin preserved the therapeutic effect while reducing cytotoxicity, reinforcing the clinical relevance of appropriately modifying the active ingredients in the original formula.

Taken together, these findings support the hypothesis that the strategic incorporation of additional components into antimicrobial pastes can enhance their therapeutic efficacy. This was also demonstrated by Kansal *et al.* (2023) [21], who showed that modifying the tri-antibiotic paste with vehicles such as 2% chlorhexidine enhanced its antimicrobial action. Under the evaluated conditions the modified tri-antibiotic paste represents a promising option for improving clinical outcome in endodontics, particularly in cases requiring prolonged and potent action against pathogenic microorganism. In this respect, Riveiro *et al.* (2022) highlighted that modified versions of the tri-antibiotic paste demonstrated durable and effective antimicrobial activity against *Enterococcus faecalis* biofilm.

Conclusion

Based on the results obtained and the comparative analysis of the evaluated formulations, it is concluded that the modified formulations paste represents a superior therapeutic alternative in terms of antimicrobial efficacy. The progressive decline observed in the bi-antibiotic paste, along with variable response of the conventional formulation, highlights the need to optimize pharmacological compositions through the incorporation of

strategic vehicles and components that promote sustained and targeted release of active ingredients.

The findings in this study, supported by multiple recent references, confirm that modifications to the formula -such as the replacement of antibiotics or vehicles- not only enhance the antimicrobial activity but also improve the penetration into the root canal system and reduce cytotoxicity. In this context, the modified tri-antibiotic paste demonstrated statistically greater efficacy against *Enterococcus faecalis* under experimental conditions. Therefore, it is recommended that this formulation be considered in future research, particularly in the evaluation of persistent infections that require a sustained and potent response against resistance microorganisms. These results contribute to the development of more effective pharmacological management techniques, reinforcing evidence-based therapeutic options for the treatment of persistent infections.

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