Original articles

Antibacterial efficacy of sodium hypochlorite and surfactant-modified chlorhexidine against *Enterococcus faecalis*: An *in vitro* study

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Received: 05/04/2026; **Accepted:** 08/07/2026; **Published:** 25/08/2026
DOI: 10.34071/jmp.2026.4.1052

Abstract

Background: *Enterococcus faecalis* (*E. faecalis*) is commonly implicated in persistent endodontic infections and has been identified as a major contributor to endodontic treatment failure. Due to the complex anatomy of the root canal system and the persistent bacterial colonization, mechanical preparation alone is often insufficient to completely eliminate bacteria and their toxins. Therefore, the present study aimed to evaluate the antibacterial efficacy of endodontic irrigating solutions.

Objective: To evaluate and compare the antibacterial efficacy of 5.25% sodium hypochlorite (NaOCl), 2% Chlorhexidine (CHX), and a surfactant-modified 2% Chlorhexidine (CHX Plus™) against *E. faecalis* biofilms in an *in vitro* model.

Materials and Methods: An *in vitro* study was conducted on 66 human mandibular premolars, which were divided into four groups: 5.25% NaOCl, 2% CHX, 2% CHX Plus™, and a negative control group (0.9% NaCl). The root canals were prepared to a master apical file size of #40 and step-back to #80. The samples were inoculated with *E. faecalis* (ATCC 29212) and incubated for 7 days to allow early biofilm formation. The teeth were then irrigated, the remaining bacteria were collected, and bacterial counts were quantified by colony-forming unit (CFU/mL) enumeration. Data were analyzed using Kruskal–Wallis and Mann–Whitney U tests ($p < 0.05$).

Results: All experimental irrigants significantly reduced the bacterial load compared to the negative control group ($p < 0.001$). Both 5.25% NaOCl and 2% CHX Plus™ demonstrated significantly higher antibacterial activity compared to conventional 2% CHX ($p < 0.05$). No statistically significant difference was observed between the NaOCl and CHX Plus™ groups ($p > 0.05$), although NaOCl showed the lowest absolute residual bacterial counts.

Conclusion: All three solutions showed high antibacterial efficacy against *E. faecalis*. However, 2% CHX was less effective than 5.25% NaOCl and 2% CHX Plus™.

Keywords: *Biofilm; chlorhexidine; Enterococcus faecalis; sodium hypochlorite; antibacterial efficacy.*

1. INTRODUCTION

Persistent endodontic infection remains a significant cause of post-treatment apical periodontitis due to the survival of resistant microorganisms within the complex root canal system. Among these pathogens, *Enterococcus faecalis* (*E. faecalis*) has been frequently associated with secondary and persistent infections [1, 2]. Its ability to penetrate deep into dentinal tubules and form organized biofilms contributes to its remarkable resistance against chemomechanical procedures [3].

Although mechanical instrumentation significantly reduces the microbial load, it cannot completely eliminate microorganisms from inaccessible anatomical regions such as lateral canals, isthmuses, and the intricacies of the dentinal tubule system. Consequently, chemical irrigation is indispensable to achieve adequate disinfection. Sodium hypochlorite (NaOCl) is currently the "gold standard" irrigant due to its broad-spectrum antimicrobial activity and unique ability to dissolve organic tissue [4]. However, concerns regarding its potential cytotoxicity to periapical tissues and its lack of substantivity remain clinically relevant. Chlorhexidine (CHX) is recognized for its broad antimicrobial spectrum and substantivity due to electrostatic interactions with dentin surfaces, providing prolonged activity [5, 6]. Nevertheless, CHX lacks tissue-dissolving capability and may exhibit limited effectiveness against biofilms due to poor penetration through the EPS matrix.

Recent advancements in irrigation research

have focused on modifying irrigant formulations to enhance penetration and antibiofilm efficacy. The incorporation of surfactants into chlorhexidine solutions aims to reduce surface tension and improve "wettability," thereby facilitating deeper penetration into the biofilm architecture and dentinal tubules. Such modifications may allow the antimicrobial agent to partially overcome the diffusion barriers traditionally associated with mature biofilms [7].

Accordingly, the present study aimed to evaluate and compare the antibacterial efficacy of 5.25% NaOCl, 2% CHX, and a surfactant-modified 2% chlorhexidine (CHX Plus™) against *E. faecalis* biofilms using a 7-day *in vitro* model. The null hypothesis considered was that there would be no significant differences in antibacterial efficacy among the evaluated irrigants.

2. MATERIALS AND METHODS

2.1. Study design

This study was a comparative in vitro experimental investigation to evaluate the antibacterial efficacy of three endodontic irrigants against an *E. faecalis* biofilm formed within instrumented human root canals.

The experimental protocol was based on the methodology described by Dametto et al. (2005), with modifications to the bacterial inoculation procedure and irrigation volume [6].

2.2. Specimen selection and preparation

Sixty-six freshly extracted human mandibular premolars obtained for orthodontic reasons were included. Teeth were selected based on the following criteria: single root with a single canal, intact crown and root structure, absence of caries, cracks, internal or external resorption, canal calcification, and fully developed apices. Root curvature did not exceed 5° according to Schneider's method [8].

Soft tissue remnants were removed mechanically. Crowns were sectioned using a diamond disc under water cooling to standardize root length at 15 mm. The working length was established 1 mm short of the apical foramen (14 mm).

The apical foramina were sealed with flowable composite resin. External root surfaces were coated with nail varnish except for the coronal access to prevent external contamination.

All specimens were sterilized in an autoclave at 121°C for 20 minutes. Sterility was verified by incubating the samples in Brain heart infusion (BHI) broth before bacterial inoculation.

2.3. Root canal instrumentation

Root canals were prepared using stainless steel K-files (Dentsply Maillefer, Ballaigues, Switzerland). Instrumentation was performed up to size #40 at working length, followed by step-back preparation to size #80 to standardize canal enlargement.

During preparation, canals were irrigated with sterile saline to avoid any antimicrobial effect before contamination.

2.4. Bacterial suspension preparation and biofilm formation

A standard strain of *E. faecalis* (ATCC 29212; American Type Culture Collection, Manassas, VA, USA) was used. After being revived, the strain was subcultured twice on Brain heart infusion agar (BHA) to obtain the F2 *E. faecalis* under aerobic conditions at 37°C.

The bacterial suspension was adjusted to McFarland standard 2 (approximately 6×10^8 CFU/mL). Each sterile canal was inoculated with 10 µL of bacterial suspension and 10 µL of BHI broth. Specimens were incubated aerobically at 37°C for 7 days to allow early-stage biofilm formation. Fresh BHI broth was replenished every 48 hours to maintain bacterial viability.

2.5. Experimental groups and irrigation protocol

The 66 specimens were randomly assigned to four groups:

- Negative control (n = 6): 0.9% sterile saline
- Group I (n = 20): 5.25% NaOCl
- Group II (n = 20): 2% CHX
- Group III (n = 20): 2% CHX Plus™

Irrigation was performed using a 30-gauge side-vented needle positioned 1 mm short of working length. Each canal received 2 mL of the assigned irrigant at a flow rate of 2 mL/min. Canals were subsequently dried with sterile paper points.

2.6. Microbiological sampling and quantification

Following irrigation, 20 µL of sterile saline was introduced into each canal. Three sterile size #30 paper points were sequentially inserted to working length and retained for 60 seconds each.

Paper points were transferred to sterile tubes containing 2 mL of saline and vortexed for 1 minute to detach adherent bacteria. Serial dilutions were prepared when necessary. Aliquots were plated onto BHA using the spread plate technique and incubated at 37°C for 24 hours.

Colony-forming units/mL (CFU/mL) were counted manually. Bacterial concentration was calculated according to the following formula:

$$\text{CFU/mL} = \frac{\text{Mean colony count} \times \text{Dilution factor} \times 1000}{a}$$

where a represents the plated volume in microliters (µL).

2.7. Outcome measures

The primary outcome measure was the post-irrigation *E. faecalis* bacterial concentration, expressed as CFU/mL. For statistical analysis, CFU values were log10-transformed.

Antibacterial efficacy was assessed by comparing the mean residual bacterial counts of each experimental group with that of the negative control, and by evaluating differences in mean residual bacterial counts among the irrigant groups.

2.8. Statistical Analysis

Statistical analysis was performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Data distribution was assessed for normality using the Shapiro–Wilk test. As data were not normally distributed, intergroup comparisons were performed using the Kruskal–Wallis test followed by the Mann-Whitney U test with Bonferroni correction for pairwise comparisons. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Evaluation of the antibacterial effect after irrigation with 5.25% NaOCl, 2% CHX, and 2% CHX Plus™

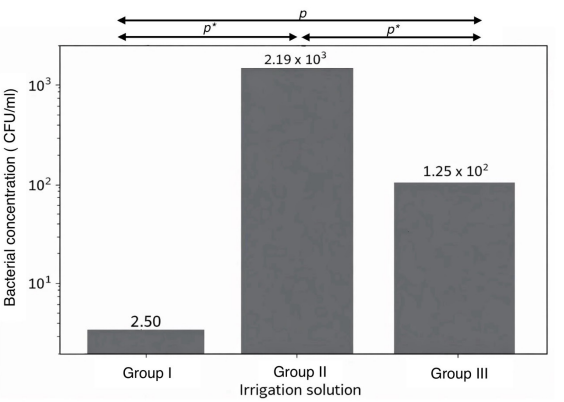
Table 1. Antibacterial effectiveness after root canal irrigation

Group	Bacterial concentration (CFU/ml)	p^*
Negative control (NaCl 0.9%)	3.21×10^6	
I	2.50	$< 0.001^a$
II	2.19×10^3	
III	1.25×10^2	

*a: Compare groups I, II, and III sequentially with the negative control group *Test Mann-Whitney U*

The negative control group indicated a residual *E. faecalis* concentration of 3.21×10^6 CFU/mL after the irrigation. Groups I, II, and III showed significant reductions in bacterial concentration, with values of 2.50 CFU/mL, 2.19×10^3 CFU/mL, and 1.25×10^2 CFU/mL, respectively. The treatment groups differed significantly from the negative control group (Mann–Whitney U test, $p < 0.001$).

3.2. Comparison of the antibacterial effectiveness of 5.25% NaOCl, 2% CHX and 2% CHX Plus™



p < 0.05; p > 0.05*

Figure 1. Comparison of the antibacterial effectiveness of root canal irrigants

The Mann-Whitney U test demonstrated that Group I (2.50 CFU/mL) and Group III (1.25×10^2 CFU/mL) had considerably lower residual *E. faecalis* counts after irrigation compared to Group II (2.19×10^3 CFU/mL), with a statistically significant difference ($p < 0.05$). In comparison with Group III, Group I displayed a lower amount of bacteria (2.50 CFU/mL compared with 1.25×10^2 CFU/mL), yet the difference was not statistically significant ($p = 0.132$). This may be related to data variability within groups and the relatively limited sample size.

4. DISCUSSION

Persistent intraradicular infection is primarily associated with structured biofilm communities rather than planktonic microorganisms. *E. faecalis* has been consistently identified in secondary and post-treatment endodontic infections and is

strongly associated with persistent periradicular lesions [1, 2]. Its ability to invade dentinal tubules, tolerate nutrient deprivation, and survive under harsh environmental conditions contributes to its persistence after chemomechanical preparation. Furthermore, biofilm architecture provides an additional layer of protection through extracellular polymeric substance (EPS) production, which restricts antimicrobial diffusion and enhances bacterial stress-response mechanisms [3].

The present findings demonstrated that both 5.25% NaOCl and 2% CHX Plus™ significantly reduced bacterial counts compared with conventional 2% CHX. NaOCl remains highly effective due to its combined antimicrobial and tissue-dissolving properties [9]. Its mechanism involves oxidative degradation of bacterial cell components, saponification of fatty acids, and disruption of the biofilm matrix [9]. Experimental studies have shown that increasing NaOCl concentration enhances its ability to eliminate *E. faecalis* within dentinal tubules [10]. Additionally, systematic evidence confirms superior antimicrobial performance of NaOCl compared with CHX during root canal disinfection [11]. The substantial bacterial reduction observed in the NaOCl group in the present study is therefore consistent with existing literature. Similar findings were reported by Jeansonne and White (1994), who demonstrated that both 5.25% NaOCl and 2% CHX significantly reduced *E. faecalis* counts compared with saline irrigation, with no significant difference between the two irrigants [12]. Berber et al. (2006) also reported that higher NaOCl concentrations improved bacterial elimination within dentinal tubules, supporting the strong antibacterial performance observed in the present study [10].

Chlorhexidine, in contrast, exerts its antimicrobial action primarily through membrane disruption and cytoplasmic precipitation [13]. Its substantivity, resulting from electrostatic interaction with dentin surfaces, allows prolonged antimicrobial activity after irrigation [6]. However, CHX lacks the ability to dissolve organic tissue, which may limit its effectiveness in disrupting early-stage biofilms [9]. Moreover, diffusion through the EPS matrix of early-stage biofilms may be restricted, reducing its antibacterial penetration [11, 14]. These factors likely explain the significantly higher residual bacterial counts observed in the conventional CHX group. The present findings are also in agreement with the study by Bhasin et al. (2019), in which 2% CHX showed lower antibacterial efficacy against *E. faecalis* compared with 5.25% NaOCl in an in vitro biofilm model [15]. However, some previous studies have reported comparable antibacterial efficacy between CHX and NaOCl, which may be related to differences in irrigation protocols, contact time, bacterial sampling techniques, and stages of biofilm development [12, 16].

The improved antibacterial performance of CHX Plus™ may be attributed to the incorporation of surfactants, which reduce surface tension and enhance wettability. Reduced surface tension may facilitate deeper penetration into dentinal tubules and improved contact with biofilm structures. Previous studies have demonstrated that CHX combined with cetrimide significantly enhances biofilm eradication compared with conventional CHX solutions [17]. Similarly, Önçağ et al. (2003) demonstrated that modified CHX formulations containing surfactant components exhibited rapid antibacterial activity against *E. faecalis* and lower cytotoxicity compared with NaOCl [16]. Surfactants may disrupt hydrophobic interactions within the EPS matrix, increasing antimicrobial diffusion and improving mechanical dislodgment of biofilm aggregates. This mechanism may explain the comparable antibacterial efficacy observed between CHX Plus™ and NaOCl in the present model.

Although NaOCl demonstrated strong antimicrobial performance, concerns regarding potential cytotoxicity and periapical tissue damage in cases of irrigant extrusion remain clinically relevant [9]. The comparable antibacterial activity observed with CHX Plus™ suggests that modified CHX formulations may represent a viable alternative in selected clinical scenarios. Nevertheless, it must be emphasized that NaOCl retains the unique advantage of organic tissue dissolution, which was not evaluated in the present study.

Several limitations should be considered when interpreting the present findings. The study employed a mono-species 7-day *E. faecalis* biofilm model, which likely represents an early/intermediate-stage biofilm rather than a fully mature biofilm. Consequently, the model may not completely reproduce the structural complexity, antimicrobial resistance, and polymicrobial interactions observed in clinically established endodontic biofilms. In addition, the in vitro conditions cannot fully simulate the dynamic environment of the root canal system in vivo. Future studies incorporating multispecies mature biofilm models, longer incubation periods, dentinal tubule penetration analyses, cytotoxicity assessment, and in vivo experimental designs are recommended

to further evaluate the clinical applicability of surfactant-modified CHX formulations.

5. CONCLUSION

Within the limitations of this in vitro study, 5.25% Sodium hypochlorite and 2% Chlorhexidine Plus™ showed superior antibacterial efficacy against *E. faecalis* biofilms compared with conventional 2% Chlorhexidine. Further studies using clinically relevant mature biofilm models are warranted.

Acknowledgements: We would like to thank our colleagues in the Department of Microbiology at

University of Medicine and Pharmacy, Hue University, for their contributions to this study.

Conflict of interest: The authors have no conflict of interest.

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