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ORIGINAL RESEARCH

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Elimination of *E. faecalis* from primary root canals using a non-instrumentation technique and rotary systems: an *ex-vivo* study

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Abstract

Background: Biofilm removal plays a key role in the long-term success of root canal therapy. This *ex-vivo* study aimed to compare the antibacterial efficacy of two rotary instrumentation systems (ProTaper Ultimate and Pro AF Baby Gold) and an irrigation-based non-instrumentation endodontic treatment (NIET) in eliminating *Enterococcus faecalis* (*E. faecalis*) biofilms from extracted mandibular second primary molars. **Methods**: A total of 45 extracted mandibular second primary molars were used. Only teeth with at least two-thirds of the root length intact and without structural anomalies were included. Five teeth served as a negative control group, while the remaining 40 were contaminated with *E. faecalis* and randomly assigned to four groups (n = 10) based on the preparation technique. Group 1: irrigation-only NIET; Group 2: ProTaper Ultimate; Group 3: Pro AF Baby Gold; and a positive control group (no instrumentation or irrigation) comprised the study groups. After treatment, biofilm samples were collected, and bacterial load was quantified by colony-forming unit (CFU) counting. Data were analyzed with the Kruskal-Wallis H test with Dunn's multiple comparisons for *post hoc* pairwise testing and the Mann-Whitney U test. Statistical significance level was set at 0.05. **Results**: The median bacterial counts were 7.99 in the control group, 6.81 in Group 1, 4.73 in Group 2, and 0.00 in Group 3. Group 1 showed no significant difference from the control group ($p = 0.3985$), but differed significantly from Group 2 ($p = 0.0031$) and Group 3 ($p < 0.0001$). Both rotary systems effectively reduced *E. faecalis* biofilms, with the highest efficacy observed in Group 3. In contrast, NIET provided limited bacterial reduction. **Conclusions**: While NIET may be useful in pediatric patients, its limited antibacterial efficacy highlights the need for further improvement before it can be considered a reliable clinical alternative.

Keywords

Enterococcus faecalis; Primary molars; Root canal treatment; Non-instrumentation endodontic treatment

1. Introduction

The optimal pulpectomy procedure for primary teeth is initiated by the comprehensive elimination—or, at the very least, the profound suppression—of the polymicrobial biofilm residing within the root canal system. *Enterococcus faecalis* (*E. faecalis*) has been recovered from almost four fifths of refractory cases and has been shown to thrive by penetrating dentinal tubules, enduring alkaline stress and surviving prolonged nutrient deprivation, thus establishing itself as the benchmark organism for *in vitro* disinfection studies [1]. *E. faecalis* is a Gram-positive, facultative anaerobic bacterium frequently implicated in persistent endodontic infections, with its prevalence reported in up to 77% of retreatment cases [2, 3]. On one hand, its remarkable ability to invade dentinal tubules, form robust biofilms, and survive under harsh environmental conditions contributes to its persistence within the root canal system [3]. On the other hand, the ribbon- or oval-shaped canals, accessory ramifications, and ongoing physiologic resorption that are characteristic of primary molars limit instrument penetration and impede the formation of a hermetic seal. Consequently, purely mechanical debridement rarely achieves complete microbial control [4, 5]. These anatomic variations, in conjunction with the behavioural challenges inherent to treating younger or special needs patients, have precipitated a biologically driven shift from the traditional dictum "instruments clean, irrigants merely flush" toward strategies that prioritise chemical disruption of biofilms while preserving dentine [6, 7].

In the treatment of pediatric patients, clinicians often face behavioral management challenges, especially in anxious,

fearful, or uncooperative children. Minimizing treatment duration and invasiveness is, therefore, essential in pediatric endodontics. The concept of a non-instrumentation technique for minimally invasive cleaning of the root canal system was first introduced in 1993 by Lussi *et al.* [8] In recent years, increasing emphasis has been placed on the concept that effective root canal cleaning may not rely solely on mechanical debridement, and that the neutralization of intracanal contents can be achieved through the use of irrigants and intracanal medicaments alone [9, 10]. In this context, recently, Non-Instrumentation Endodontic Treatment (NIET) is predicated on this minimalist philosophy, whereby canals are left uninstrumented and disinfected with potent irrigants such as Sodium hypochlorite or Chlorhexidine, often supplemented by intracanal medicaments like triple antibiotic or chloramphenicol-tetracycline-zinc oxide (CTZ) pastes. In the present study, NIET refers specifically to an irrigation-based, antibiotic-free protocol and should not be considered equivalent to Lesion Sterilization and Tissue Repair (LSTR) techniques employing intracanal medicaments. Previous systematic reviews have reported bacterial reductions approaching 100%, with clinical outcomes comparable to those of conventional pulpectomy treatment [7, 11]. Furthermore, the chair time is markedly reduced, especially in pediatric dental patients [12]. In this context, in the preceding decade, there has been a notable emergence of pediatric Nickel–Titanium (NiTi) rotary systems (for instance, Kedo S, Kedo SG Blue, Pro AF Baby Gold) that have been developed with shortened working lengths, progressive tapers, and heat-treated alloys. Although ProTaper Ultimate was originally developed for permanent teeth, its inclusion in the present *ex-vivo* study was intended to evaluate the cleaning efficacy of a contemporary heat-treated NiTi system under controlled conditions. This comparison was designed to provide a benchmark against pediatric-specific rotary files and does not imply routine clinical recommendation for primary teeth. These systems have been produced to enable safe negotiation of narrow and resorbing canals. Scoping reviews and *ex-vivo* studies consistently demonstrate that these files reduce instrumentation time by 25–75% and can decrease *E. faecalis* counts by ≥90% in comparison with manual approaches [13, 14]. Despite the promise of both approaches, direct headtohead evidence comparing NIET with contemporary rotary systems in terms of quantitative *E. faecalis* elimination in primary teeth remains scant. The present *ex-vivo* study, therefore, seeks to bridge this knowledge gap and inform a clinically balanced protocol that reconciles minimal invasiveness with rigorous microbiological goals.

To date, no *ex-vivo* study has evaluated NIET specifically in primary teeth. Moreover, in dentistry literature, previous studies especially have focused on permanent teeth regarding minimally invasive endodontic approaches. Based on the above argument, the present *ex-vivo* study aimed to assess and compare the antibacterial performance of two rotary instrumentation systems (ProTaper Ultimate and Pro AF Baby Gold) and a NIET approach in the elimination of *E. faecalis* biofilms from extracted mandibular second primary molars. The null hypothesis (H0) was that there would be no statistically significant difference among the study groups regarding the residual bacterial load.

2. Materials and methods

2.1 Study design and checklist

This research was an *ex-vivo* laboratory study conducted on primary teeth extracted from pediatric dental patients. In addition, the current study was conducted following the Preferred Reporting Items for Laboratory studies in Endodontology 2021 (PRILE-2021) guidelines for reporting laboratory studies in Endodontology [15]. Study procedures were conducted between February 2025 and July 2025.

2.2 Sample size analysis

The sample size was determined using G*Power version 3.1.9.6 (Heinrich Heine University, Düsseldorf, NRW, Germany), based on the effect size reported in a previous study [2], with a statistical power of 0.80 and a significance level of $\alpha = 0.05$. Accordingly, the minimum required sample size was calculated as 40.

2.3 Sample selection criteria and storage media

A total of 45 mandibular second primary molars, extracted due to infection or extensive caries, were collected. To minimize anatomical variability, only teeth with at least two-thirds of their original root length intact were included. Teeth presenting structural anomalies, such as root fractures, dentinal microcracks, partial or complete intracanal calcifications, or resorption cavities on the internal or external root surfaces, were excluded from the study. After extraction, any residual soft tissue and debris were removed using a periodontal curette. The teeth were then stored in distilled water containing 0.1% thymol crystals at room temperature until the initiation of the experimental procedures. This solution was used to inhibit microbial growth during the storage period without affecting the tooth structure.

2.4 Procedure and tooth preparation

Access to the root canal system was achieved through the crown using a water-cooled round diamond bur (Meisinger, 801G-coarse, Hager & Meisinger GmbH, Neuss, Germany). The endodontic working length was measured using a size 15 K-file to be 1 mm shorter than the root apex on periapical radiographs. Pulpal tissue was removed with a size 15 K-file (VDW, Munich, Germany). The apical foramen was sealed using a flowable composite resin (3M ESPE, Bayem, Germany). Following these procedures, all specimens were sterilized in an autoclave at 121 °C for 15 minutes and stored in a dry environment until use.

2.5 Bacterial species and inoculum preparation

E. faecalis ATCC 29212 (American Type Culture Collection, Manassas, VA, USA), obtained from the microbiology laboratory's culture collection, was cultivated overnight in tryptic soy

agar at 37 °C under aerobic conditions. In order to prepare the inoculum, colonies from the active bacterial culture were collected into a glass tube containing 5 mL of sterilised phosphate-buffered saline (PBS) until a bacterial suspension equivalent to 2.0 on the McFarland scale was obtained (approximately 6.0×10^8 colony-forming unit (CFU)/mL). The prepared bacterial suspension was then mixed with an equal volume of brain heart infusion culture medium to achieve a concentration of approximately 3.0×10^8 CFU/mL (1.0 MacFarland scale). In order to ensure the viability of the *E. faecalis* culture necessary for consistent biofilm development, fresh bacterial cultures were prepared on a daily basis for 48 hours [16, 17].

2.6 *Ex-vivo* root canal infection with *E. faecalis*

Under sterile conditions, the teeth were staged in a 24-well plate, and the bottom of the plate was coated with sterile orthodontic wax. The root canals of the specimens were then inoculated with a 10 μL volume of the prepared *E. faecalis* bacterial suspension, employing aseptic techniques to ensure sterility. Following inoculation, the samples were subjected to an incubation period of 48 hours at a temperature of 37 °C. At the 24th hour of incubation, 10 μL of freshly prepared bacterial suspension was added to the root canals [18].

2.7 Procedure of endodontic treatment

A total of 40 specimens were randomly assigned to four groups according to the root canal preparation technique and treatment protocol:

Group 1 (Irrigation-only NIET) (n = 10): No mechanical canal instrumentation was performed; canals received only 10 mL of 2.5% Sodium hypochlorite (NaOCl) delivered by manual syringe irrigation. This group was included as a mechanistic baseline to isolate the antibacterial effect of irrigant delivery alone (without canal shaping, intracanal medicament or activation) and was not intended to represent a definitive clinical "irrigate-and-obturate" protocol.

Group 2 (Protaper Ultimate) (n = 10): Root canal preparation was performed using the ProTaper Ultimate system (Dentsply Sirona, Bensheim, HE, Germany) with the Slider (016/0.002v), Shaper (020/0.004v), and finishing files up to size 25 (F1: 020/0.007v; F2: 025/0.008v). 10 mL of 2.5% NaOCl was used during instrumentation for irrigation and the final rinse.

Group 3 (Pro AF Baby Gold) (n = 10): Root canal preparation was performed using Pro AF Baby Gold file system (Dentalyze, Mumbai, India) B0 (15/0.10), B1 (20/0.04), and B2 (25/0.04) NiTi rotary files, and 10 mL of 2.5% NaOCl was used during instrumentation for irrigation and the final rinse.

Control Group (n = 10): Infected specimens that did not receive any mechanical instrumentation or irrigation. This group was used to determine the baseline bacterial load (CFU/mL).

For Group 1, irrigation was performed exclusively as the final irrigation step, utilising 10 mL of 2.5% sodium hypochlorite. For Groups 2 and 3, irrigation was performed using 10 mL of 2.5% sodium hypochlorite between each mechanical instrumentation and during the final irrigation. As previously mentioned, no irrigation procedure was performed in the control group. The files were activated by Endo-Mate DT motor (NSK, Tochigi, Japan) at 250 rpm and 0.8 N/cm maximum torque. No obturation step was performed in any group because the primary endpoint of this *ex-vivo* study was immediate intracanal bacterial reduction.

In addition to the experimental and positive control groups, a negative control group (n = 5) composed of sterilized, non-inoculated specimens was incorporated to confirm aseptic conditions and serve as a baseline for contamination control.

2.8 Biofilm collection from the root canals

The method for biofilm collection was adapted from a previously described protocol by Goulart *et al.* [19] (2024), with modification. After completing irrigation procedures at the end of incubation, root canals were washed with sterilized PBS to help remove any remaining planktonic cells. Samples were transferred to 1.5 mL Eppendorf tubes using sterile tweezers, and 500 μL of sterile PBS was added. The specimens were vigorously vortexed at maximum speed for 1 min and then sonicated in an ultrasonic bath at room temperature for 8 min. Subsequent to this, centrifugation was conducted at 700×g for 1 min. For the CFU/mL calculation, samples were serially diluted four times at a 10-fold dilution and spread on tryptic soy agar and incubated for 24 hours at 37 °C. After incubation, the colonies formed on the agar surface were counted, and CFU/mL values were calculated for the control and experimental groups.

2.9 SEM analysis

The presence of bacteria on root canal surfaces was examined using a scanning electron microscope (SEM) (TESCAN GAIA3 Triglav™, Brno, Czech Republic). The roots were longitudinally sectioned into two halves using sharp diamond disks under water cooling. Subsequently, the samples were vacuum-dried, mounted on aluminum stubs, and sputter-coated with a 135 Å layer of gold-palladium alloy (80% Au, 20% Pd) using a sputter coater (SBC-800, Karaltay Beijing Instruments, Beijing, China) prior to SEM imaging. The operating voltage (EHT) was set to 15.00 kV. The magnification ratio was 10.0 kx. The detector was used as Secondary Electron Detector. The scale bar is indicated as 20 μm in the image. The working distance was 10.0 mm, and the scanning mode was recorded as "Signal A = SE1". These parameters provided a standard SEM imaging that allowed detailed observation of surface morphology.

2.10 Blinding

The specimens included were assigned sequential numbers and coded by an independent operator who was not involved in either the treatment or the assessment of outcomes. Consequently, the microbiologist (BA) who collected and quantified the biofilm samples and the examiner (AÖ) who conducted the SEM analyses remained unaware of the group allocation throughout the study. The code key was unsealed only after all measurements had been completed, thereby providing a double-blind design that minimised assessment bias.

2.11 Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 10.5.0, GraphPad Software, San Diego, CA, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. Since two of the treatment groups did not meet the assumption of normality, non-parametric statistical tests were applied. Comparisons among the four groups (control and three treatment groups) were conducted using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test for pairwise group analyses. The results are presented as medians with minimum and maximum values. A p-value of less than 0.05 was considered statistically significant. Adjusted p-values from Dunn's test are reported to indicate significance between groups.

3. Results

The bacterial load within the dentinal tubules was quantified as $\log_{10}$ CFU/mL and compared among the control and experimental groups. All five negative control specimens exhibited 0 CFU, confirming aseptic conditions and serving as the baseline bacterial load for the study. The median bacterial count was 7.99 (6.93–9.41) in the positive control group, 6.81 (5.09–8.51) in Group 1, 4.73 (0.00–8.63) in Group 2, and 0.00 (0.00–5.07) in Group 3. The bacterial concentration across the groups is presented in Table 1 and Fig. 1. Kruskal-Wallis analysis revealed a statistically significant difference among the groups ($p < 0.0001$). Subsequent pairwise comparisons using Dunn's multiple comparisons test demonstrated that both Group 2 and Group 3 had significantly lower bacterial counts compared with the control group ($p < 0.0001$ for both). Although Group 1 did not show a significant difference from the control group ($p = 0.3985$), it differed significantly from both Group 2 ($p = 0.0031$) and Group 3 ($p < 0.0001$). No significant difference was observed between Group 2 and Group 3 ($p = 0.2464$). These findings suggest that Group 2, and particularly Group 3, were effective in reducing the intracanal bacterial load. Fig. 2 presents experimental details, including the culture plates showing bacterial growth following the interventions and SEM imaging. SEM images of the control group and the experimental groups are shown in Figs. 3,4, respectively.

TABLE 1. Bacterial concentrations ($\log_{10}$ CFU/mL) expressed as median and range (minimum–maximum) in control and treatment groups.

Groups	Median	Min	Max
Group 1 (NIET)	6.81	5.09	8.51
Group 2 (ProTaper Ultimate)	4.73	0.00	8.63
Group 3 (Pro AF Baby Gold)	0.00	0.00	5.07
Positive control	7.99	6.93	9.41
p		<0.0001	

Min: minimum; Max: Maximum; NIET: Non-Instrumentation Endodontic Treatment. $p < 0.05$ statistical difference.

4. Discussion

The present *ex-vivo* study evaluated the NIET as a minimally invasive alternative to conventional root canal preparation. This approach may be particularly useful in cases involving young, anxious, or uncooperative paediatric patients, in which treatment duration and patient comfort are critical considerations. Based on the obtained data, the overall null hypothesis was rejected; *post hoc* analysis showed that both rotary systems differed significantly from the control, whereas the non-instrumentation technique did not. Despite achieving only minimal bacterial reduction, the NIET was found to be significantly less effective than both rotary instrumentation systems. Furthermore, no statistically significant difference was observed between the NIET and the positive control group. The findings of this study indicate that, under the conditions of the present study, the use of irrigation alone appears to be inadequate for the effective elimination of mature biofilms from the root canal systems of primary teeth.

It is important to emphasize that the NIET protocol evaluated in the present study was limited to irrigation alone and did not include intracanal antibiotic medicaments, such as those used in LSTR techniques. This design was intentional and aimed to isolate the antibacterial effect of chemical irrigation without mechanical preparation or adjunctive medicaments. Therefore, the findings should not be interpreted as representative of all NIET or LSTR-based protocols, but rather as evidence regarding the limitations of irrigation-only approaches in primary teeth.

It is acknowledged that an irrigation-only approach without instrumentation or intracanal medicaments is not currently considered a standard clinical protocol in pediatric endodontics. However, with the increasing emphasis on minimally invasive strategies and biologically driven disinfection concepts, it remains clinically relevant to explore whether simplified non-instrumentation approaches could represent a future direction under optimized conditions. In this context, Group 1 was intentionally designed as an exploratory *in vitro* model to evaluate the baseline antibacterial potential of irrigation alone in primary teeth, rather than to replicate an established clinical treatment protocol. Irrigation alone should not be interpreted as a definitive clinical approach for necrotic primary teeth. Rather, the irrigation-only arm serves as a laboratory control representing the minimum level of chemical decontamination achievable in the absence of instrumentation or adjunctive measures, thereby enabling quantification of the incremental benefit of rotary shaping under standardized conditions.

In this study, 2.5% NaOCl was selected as the irrigant due to its well-documented bacteriostatic properties, ability to dissolve organic tissues, and effective penetration into dentinal tubules [2, 20]. In primary molars, anatomical features, such as narrow, curved canals, numerous accessory canals, and the close proximity of the developing permanent tooth bud, limit both mechanical access and irrigant flow [21]. The combination of these factors, in the absence of mechanical instrumentation, may provide a rationale for the observed limitations in antibacterial efficacy within the NIET group. Furthermore, although NaOCl has been demonstrated to penetrate dentinal tubules to depths of approximately 300 μm, *E.*

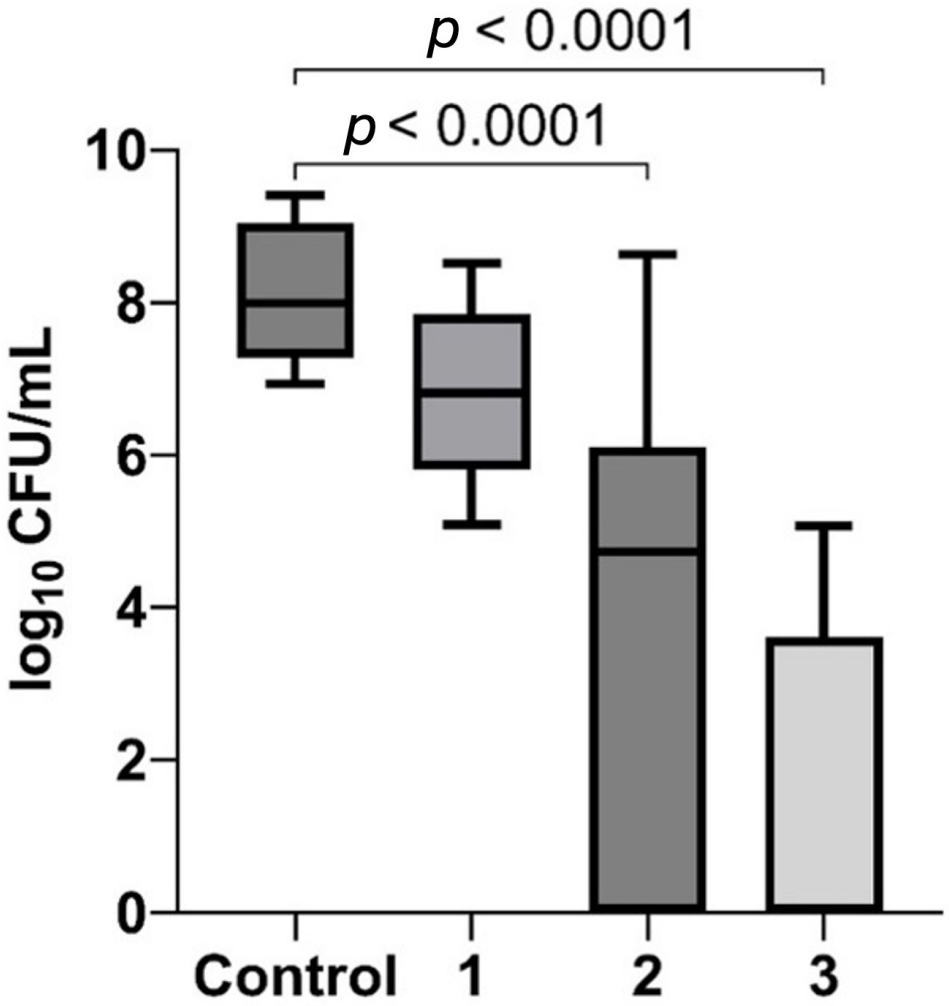

FIGURE 1. Comparison of bacterial reduction in root canals treated with different protocols. Data are presented as median values with minimum and maximum. Statistical significance was assessed by Kruskal-Wallis test with Dunn's *post hoc* test. CFU: colony-forming unit.

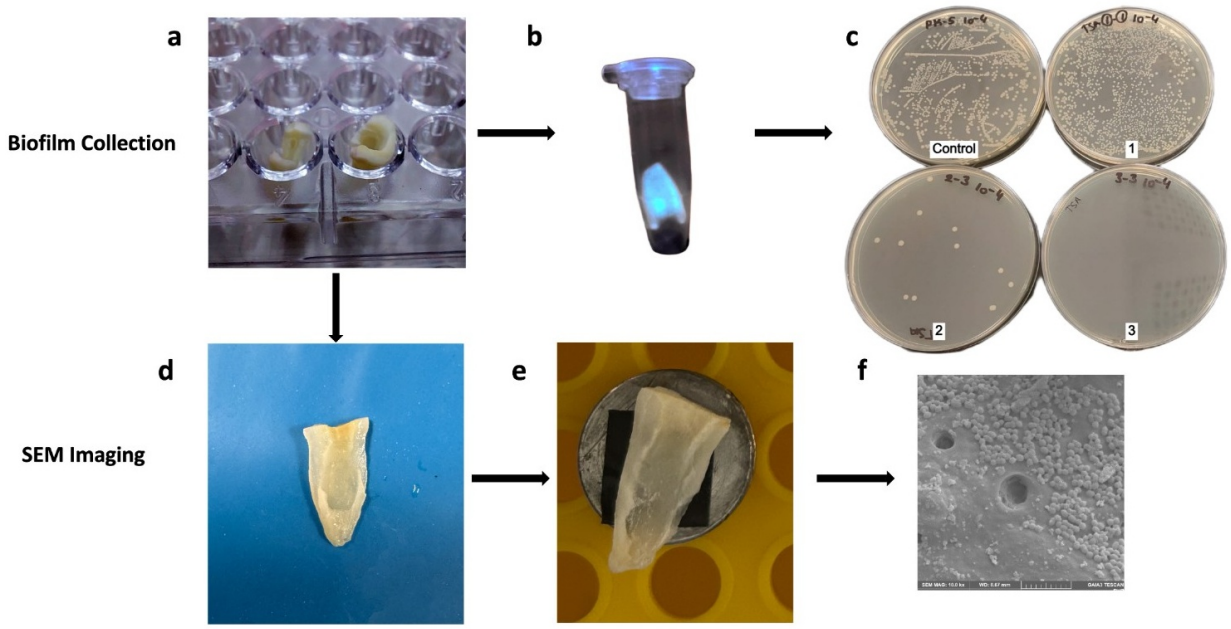

FIGURE 2. The experimental flow chart of the study. (a) the root in the plate, (b) the root in the Eppendorf tube before centrifuging, (c) Bacterial culture plates from Groups I, II, III, and the control group following the interventions, (d) the half of the root after seperation, (e) the sample on the aluminum stub, (f) The obteined SEM image. SEM: scanning electron microscope.

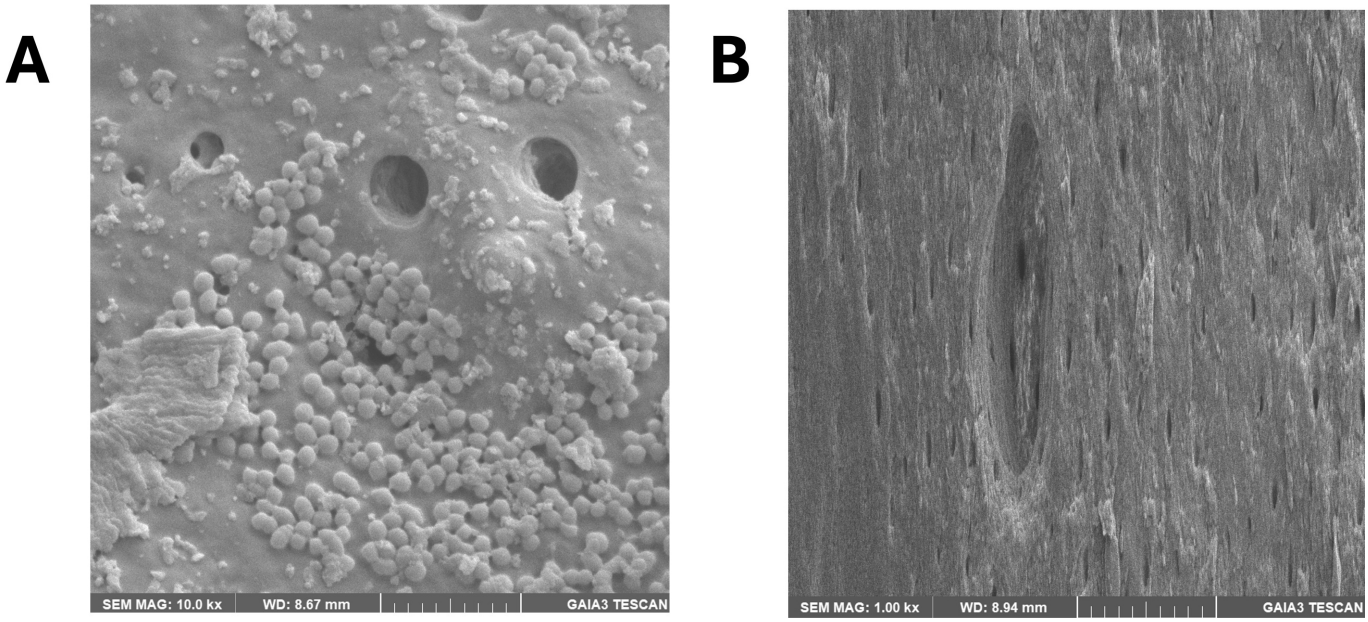

FIGURE 3. SEM images of the control groups: (A) positive control at 10.0 kx and (B) negative control at 1.00 kx magnification. (A) The dentin surface exhibits multiple open tubules surrounded by abundant spherical, microorganism-like particles and amorphous debris, consistent with bacterial biofilm presence. (B) The dentin surface shows clean tubule morphology with no observable microbial aggregates, debris, or biofilm-related structures.

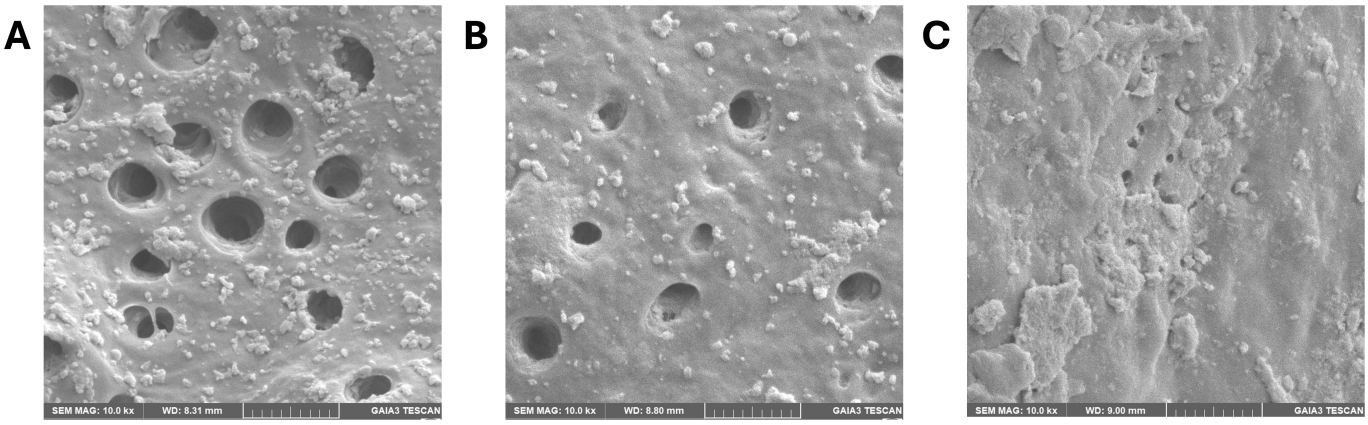

FIGURE 4. SEM images of (A) Group 1 (NIET), (B) Group 2 (ProTaper Ultimate), and (C) Group 3 (Pro AF Baby Gold) under 10.0 kx magnification. Dentin tubules appear as round openings on the dentin surface. The amorphous, granular, and irregular structures observed around the tubule orifices and across the surface represent components of the bacterial biofilm, including extracellular polymeric substances (EPS) and microorganism-like particles. These biofilm-associated formations appear as uneven layers, clustered particulate accumulations, or dense deposits partially occupying the tubule openings. In some regions, organic–inorganic debris, bacterial aggregates, and matrix-like structures are evident, reflecting the morphological characteristics of microbial colonization.

faecalis has been reported to invade tubules as deep as 500–1000 μm [17, 19]. Consequently, irrigation alone may be insufficient to achieve complete eradication of *E. faecalis* in infected root canals.

The use of sterilized paper points remains a common method for collecting bacterial biofilms from root canals. However, this technique primarily captures planktonic bacteria and falls short in effectively retrieving biofilm-associated microorganisms [19, 22, 23]. Moreover, it has notable limitations in accessing the full extent of the root canal system. In 2024, Goulart *et al.* [19] highlighted these limitations and introduced an alternative collection protocol involving vortex mixing, ultrasonic bath immersion, and centrifugation. This updated method demonstrated superior efficacy in biofilm retrieval compared with the conventional paper point technique [19]. In light of these findings, the current study adopted this modernized protocol to ensure more comprehensive and reliable sampling of biofilm within the root canal system.

Although CFU counting is widely regarded as the gold standard for assessing the reduction of viable and culturable *E. faecalis* in endodontic studies and allows direct comparison with the majority of previous literature, it is acknowledged

that more advanced molecular techniques (*e.g.*, quantitative polymerase chain reaction (qPCR), next-generation sequencing (NGS) or live/dead staining) could provide additional information on total bacterial DNA, viability status, and biofilm biomass [2–4, 9, 21, 24]. Nevertheless, since the clinical relevance is primarily attributed to the elimination of cultivable bacteria capable of proliferation and reinfection, and given that the monoculture model was utilised, it was determined that CFU counting was the most appropriate primary outcome measure. SEM analysis was included to support the quantitative findings with qualitative ultrastructural evidence.

While some *in vitro* studies have demonstrated that conservative instrumentation combined with activated irrigation can yield canal cleanliness comparable to that achieved with larger apical preparations [10, 25, 26], the effectiveness of a completely non-instrumentation protocol remains controversial—especially in the presence of established biofilms. Our findings align with those of Ordinola-Zapata *et al.* [9] who reported that non-instrumented canals irrigated with a multisonic device were less effective in reducing microbial load compared with canals that were conventionally prepared and ultrasonically irrigated. This outcome may have been a result of the absence of a tapered canal shape in the NIET group, which likely reduced the shear forces required for effective biofilm disruption [9].

It is also important to highlight that most of the existing studies supporting the effectiveness of conservative or non-instrumentation approaches have been conducted in permanent teeth [9, 26, 27]. Due to the anatomical and structural differences between primary and permanent teeth—such as thinner dentinal walls, more accessory canals, and ongoing physiological root resorption—the applicability of such findings to primary teeth remains uncertain [28]. These morphological characteristics may further limit the penetration and efficacy of irrigants when instrumentation is omitted.

The ProTaper rotary file system family has been evaluated in previous studies in terms of preparation capability and cleaning efficiency in primary root canals [29–31]. This study compared the NIET method and a rotary file (Pro AF Baby Gold) specifically designed for pediatric endodontic applications with the latest generation of the ProTaper Universal system. Previous studies have shown that Pro AF Baby Gold is effective for the mechanical preparation of primary teeth [32, 33]. The present findings further confirm its effectiveness in reducing the microbial load in root canals. Additionally, this study is the first to use ProTaper Ultimate in the preparation of primary teeth to evaluate its microbial cleaning efficacy. Both file systems achieved a similar level of microbial load reduction in primary root canals. However, the taper and tip dimensions of the ProTaper Ultimate system should be carefully evaluated in terms of their potential effects on danger zone within primary root canal morphology.

Lesion Sterilization and Tissue Repair (LSTR) is an alternative endodontic technique that does not involve instrumentation. It utilises various combinations of antibiotics to disinfect necrotic primary teeth with periapical lesions. The efficacy of this treatment modality in managing necrotic primary teeth, with or without periapical involvement, is contingent upon the condition of root resorption not exceeding half the root length. This assertion is particularly salient in cases necessitating short-term space maintenance [7]. Despite LSTR being classified as a NIET, it deviates from the methodology employed in our study with regard to both its indications and the clinical application protocol. Consequently, a comparison of the outcomes of the two methods would be ill-advised.

It is imperative to acknowledge the limitations of this study. First, while allowing for standardisation and controlled conditions, the *ex-vivo* study design did not fully replicate the complex biological environment of the oral cavity. Factors such as immune responses, salivary conditions, and host–microbe interactions, which may influence disinfection efficacy, were not represented in this model. Also, only a single bacterial species, *E. faecalis*, was used to create a mono-species biofilm. Although *E. faecalis* is a clinically relevant and highly resistant endodontic pathogen, root canal infections *in vivo* are typically polymicrobial in nature, and the results may not fully reflect clinical scenarios involving mixed-species biofilms. In this context, future studies should incorporate polymicrobial models to better mimic clinical infection complexity. A further explanatory factor for the limited disinfection in the irrigation-only group is the apical vapor lock phenomenon. In the present model, the apical foramen was sealed to create a closed-end system, which is prone to entrapping an apical air bubble and restricting irrigant exchange at the apical third of the root canal system. Under such conditions, manual syringe irrigation (particularly without canal enlargement) may be unable to disrupt the vapor lock and generate sufficient shear stress for mature biofilm removal. This supports the rationale that non-instrumentation strategies typically require enhanced fluid dynamics through irrigant activation (for example ultrasonic/sonic/multisonic approaches) to improve apical replacement and biofilm disruption.

In addition, although rotary systems were evaluated, no obturation or post-treatment evaluation of reinfection or healing was performed. Long-term outcomes, such as bacterial reinfection, root resorption, or treatment success over time, were not assessed in the current study and should be addressed in future study designs. A further limitation was that NaOCl was the only irrigant used in this study, which limits the applicability of the findings to other irrigation protocols. Further designs should compare NaOCl with alternative or adjunctive irrigants to determine the most effective and biocompatible protocol for primary teeth. Additionally, evaluating different irrigant volumes, irrigating time, and concentrations may offer valuable insights into optimizing disinfection outcomes. Also, while the present study concentrated on extracted mandibular second primary molars, it is acknowledged that results may vary with different tooth types or canal morphologies. Consequently, it is imperative to exercise caution when generalising the findings to other primary teeth types. Future studies should incorporate additional tooth types to assess whether anatomical differences and ramifications influence disinfection outcomes. Moreover, it is important to distinguish the simple irrigation-based NIET protocol used in this design from LSTR techniques, which employ antibiotic pastes and follow different clinical indications and procedural steps. Future research should explore how NIET protocols can be optimized, including adjustments to irrigant parameters or the use of activation systems, to enhance their antimicrobial potential and broaden their applicability in

paediatric endodontics.

In view of the limitations of the present study, future research should concentrate on optimising NIET by examining various parameters with the potential to enhance their antimicrobial efficacy. It is recommended that subsequent research consider additional variables, including but not limited to: different tooth types, root canal anatomy, instrumentation systems, and microbial species. These factors have been demonstrated to have significant influences on the effectiveness of disinfection protocols. Such refinements have the potential to enhance the clinical efficacy of NIET and to expand its applicability to a wider range of paediatric endodontic cases.

5. Conclusions

The NIET appears to be a theoretically advantageous approach in pediatric endodontics due to its simplicity, speed, and reduced risk of iatrogenic damage. However, under the conditions of this *ex-vivo* study, an irrigation-only NIET protocol, applied without mechanical preparation or medicament use, was insufficient to effectively reduce the bacterial load in infected primary root canals. In contrast, both rotary systems showed superior antibacterial efficacy when combined with a standard irrigation protocol. These findings suggest that mechanical preparation remains essential for adequate disinfection. Further studies are warranted to explore optimized protocols for NIET and assess its clinical applicability in pediatric dentistry.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available on request from the corresponding author.

AUTHOR CONTRIBUTIONS

BC and AD—designed the study. MO, AO, EO and BA—performed the experimental procedure. AO and BA—analyzed the data. AD and MO—wrote the main manuscript. All authors checked the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval for the current study was obtained from Lokman Hekim University Faculty of Dentistry Ethics Committee on 30 May 2025 with the decision number 2025/143. As the study procedures involved the use of extracted primary molar teeth from the pediatric dental patients, verbal and written informed consent forms were approved by all the patients and their parents/legal guardians. Also, the study protocol was conducted in accordance with the ethical principles for medical research (involving human participants, including research using identifiable human material or data) of the World Medical Association (WMA) Declaration of Helsinki.

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Not applicable.

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The financial aspects of this study were provided by the authors; there is no external source.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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