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Original Article

Influence of Biomaterial–Dentin Interface, Aging Medium, and Duration on Bacterial Biofilm Proliferation and Viability

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ABSTRACT

As time progresses, the junction between dentin and restorative biomaterials deteriorates, facilitating the passage of saliva, tissue fluids, and bacteria between the filling or restorative substance and the dentin surface. The present investigation explored how long-term exposure to simulated human salivary/bacterial/blood esterases (SHSE) affects the development and survival of Enterococcus faecalis biofilms formed along the dentin interface when different endodontic materials are employed. Extracted human anterior teeth were mechanically prepared and obturated with gutta-percha in combination with one of four materials: a self-cured resin composite (Bisfil™ 2B, Bisco, Schaumburg, IL, USA) paired with either a self-etch adhesive (EasyBond) or a total-etch adhesive (Scotchbond™, 3M, Saint Paul, MN, USA), an epoxy resin-based sealer (AH Plus®, Dentsply Sirona, York, PA, USA), or a bioceramic sealer (EndoSequence® BC Sealer™, Brasseler USA, Savannah, GA, USA). Samples were then subjected to accelerated aging in SHSE or phosphate-buffered saline (PBS) for intervals of up to 360 days, after which stable E. faecalis biofilms were cultivated. The extent and vitality of bacterial growth within the interfacial zones were examined using confocal laser scanning microscopy coupled with live/dead viability staining. Data analysis was conducted using a three-way ANOVA with Scheffé's post hoc comparisons. At baseline, all materials exhibited comparable biofilm penetration depths ($p > 0.05$). Prolonged aging led to significantly greater bacterial infiltration across all material types ($p < 0.05$). SHSE exposure intensified interfacial biofilm penetration for the TE, SE, and BC materials ($p < 0.05$), while the AH group remained unaffected. In unaged conditions, BC showed the smallest proportion of live bacterial cells, followed by AH, TE, and SE ($p < 0.05$). Overall, the proliferation and viability of interfacial E. faecalis biofilms were influenced by the type of biomaterial, the composition of the aging environment, and the duration of aging.

Keywords: Interface, Biofilm proliferation, Biomaterial–dentin interface, Enterococcus faecalis, Salivary enzymes, Epoxy-resin sealer

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Introduction

Bacterial biofilms are recognized as the main etiological factor behind root canal infections, as microorganisms can endure within the canal system despite thorough chemomechanical debridement, often recolonizing to their original levels [1]. Enterococcus faecalis is frequently associated with persistent endodontic infections [2], primarily due to its ability to form resilient biofilms, degrade dentinal collagen and methacrylate-based resins, and survive under hostile environmental conditions [2–4]. In endodontic therapy, root canal sealers are employed alongside a core filling material to occupy the interface between the canal wall and the core, thereby trapping residual bacteria and preventing microbial re-entry or biofilm formation. An optimal sealer should combine antimicrobial efficacy, dimensional stability, effective sealing capability,

mechanical strength, and durability against tissue fluids [5].

Epoxy-resin-based sealers, such as AH Plus (AH), have long been regarded as the clinical benchmark due to their superior handling, physicochemical properties, and biocompatibility. Nevertheless, their weak bonding to core materials and reduced antimicrobial effectiveness after polymerization remain drawbacks [6]. A newer alternative, bioceramic (BC) sealers, is based on hydraulic calcium silicate cements that utilize dentinal moisture to complete their setting reaction and form hydroxyapatite, facilitating a chemical bond with dentin [7]. Although methacrylate-based resins have been proposed for use as sealers, their clinical performance has been less favorable [8]; hence, they are now more commonly applied in post cementation [9] and restorative procedures [10]. Adhesive systems for resin composites are designed to enhance the bond between the composite and tooth substrate, improving marginal sealing in root canal restorations [8]. These adhesives fall into two main categories: total-etch (TE) systems, which remove the smear layer, and self-etch (SE) systems, which partially dissolve and modify it [11]. Both systems depend on forming a hybrid layer, a resin-infiltrated network of dentinal collagen, to achieve micromechanical retention [12].

Over time, the biomaterial–dentin interface experiences degradation, enabling passage of saliva, tissue fluids, and bacteria between the restorative or root-filling materials and the dentin substrate [12–14]. Human salivary esterases, bacterial enzymes, and neutrophil-derived esterases—either naturally present or simulated in vitro—alter the mechanical and chemical properties of this interface, accelerating its breakdown in ways that depend on the material composition [3, 14–16]. The by-products of this degradation can further influence bacterial gene regulation, protein synthesis, and even virulence potential [14, 17]. Consequently, the compromised interface can allow microbial leakage, dentin matrix degradation, and reduced bond strength [12, 18], which, in endodontic settings, may contribute to post-treatment apical periodontitis [19]. Despite these known effects in restorative contexts, such interactions have not been fully examined within endodontic systems. Therefore, it is essential to evaluate both traditional and novel sealers for their interfacial stability and resistance to degradation under conditions that replicate the oral biochemical environment.

The purpose of this study was to evaluate how long-term exposure—up to 360 days—to simulated human salivary/bacterial/blood esterases (SHSE) impacts E. faecalis biofilm growth and viability within the dentin–biomaterial interface in teeth treated with four different filling or restorative materials. Although E. faecalis is not the only species involved in endodontic infections, it serves as a well-characterized indicator organism for studying bacterial penetration in such settings. The tested materials represent key categories of endodontic sealers and restorative resins, including methacrylate-based composites with either self-etch or total-etch adhesives, an epoxy resin sealer, and a hydraulic calcium-silicate sealer. The working hypothesis proposed that biofilm proliferation and viability at the dentin interface would vary according to the biomaterial type, the aging medium, and the exposure duration, emphasizing the significance of mimicking intraoral degradative conditions when assessing endodontic material performance.

Materials and Methods

Specimen preparation

A power analysis, based on pilot data, was conducted to establish the minimum number of samples needed to detect a significant effect ($\alpha = 0.05$, $1 - \beta = 0.8$) using G*Power 3.1.9.2 software (Heinrich-Heine-Universität Düsseldorf, Germany). An effect size threshold of one unit (1 µm or 1 point difference in live/dead ratio) indicated that two teeth per group per time point would yield sufficient power; in this study, three teeth per group per time point were used.

Caries-free human anterior teeth were collected, stored at −20 °C in distilled water until use, and ethically approved by the University of Toronto (protocol #28214) [3, 12, 20]. Each tooth was examined under an operating microscope to exclude any with cracks. The crowns were sectioned off, and all endodontic procedures were conducted under aseptic conditions. Root canals were gradually enlarged to diameters of 0.9–1.5 mm using parallel drills (ParaPost®, Coltene, Cuyahoga Falls, OH, USA), followed by microscopic inspection to ensure integrity. Roots were sterilized by autoclaving, a process shown not to alter dentin properties [21]. The canals were irrigated sequentially with 5 mL of 5.25% NaOCl, 5 mL of 17% EDTA, and a final rinse of 10 mL sterile distilled water before being dried with sterile paper points. Specimens were randomly allocated to one of four experimental groups—SE, TE, AH, or BC **(Table 1)**—and obturated with gutta-percha and the corresponding sealer. All samples were stored at 37 °C in 100% humidity for 72 h to ensure complete setting.

Table 1. Description of the materials used as sealers in the experimental groups.

Group	Description
SE	Resin composite (Bisfil™ 2B, Bisco, Schaumburg, IL, USA) bonded to root dentin using self-etch adhesive (Adper™ Easy Bond, 3M, Saint Paul, MN, USA).
TE	Bisfil™ 2B bonded to root dentin using total-etch adhesive (Scotchbond™, 3M, Saint Paul, MN, USA)
AH	epoxy-resin-based sealer (AH Plus®, Dentsply Sirona, York, PA, USA).
BC	Bioceramic sealer (EndoSequence BC Sealer, Brasseler USA, Savannah, GA, USA)

A 3 mm section was cut from the coronal portion of each prepared root using a water-cooled, slow-speed diamond saw. Both apical and coronal surfaces of the obtained segments were sequentially polished with 600–1200-grit silicon carbide abrasive papers to achieve smooth, uniform surfaces [16]. To prevent any unintended bacterial penetration through the exposed dentinal tubules, all coronal dentin adjacent to the root fillings and the external cementum were sealed with a layer of clear varnish.

Specimen aging

Each material group (n = 3 per material per time point) was divided into unaged control specimens and those subjected to aging under two conditions—simulated human salivary/bacterial/blood esterases (SHSE) or phosphate-buffered saline (PBS). Samples were incubated at 37 °C and pH 7.0 for 30, 180, or 360 days. The SHSE solution was prepared by dissolving cholesterol esterase (CE) and pseudocholine esterase (PCE) in PBS, with enzymatic activities maintained at physiologically relevant levels (16 U/mL for CE and 0.01 U/mL for PCE), as previously established [18]. Throughout the incubation period, the aging media were regularly replaced to preserve enzyme activity consistent with natural salivary conditions.

Biofilm cultivation

After completing their designated aging period, the specimens were transferred to a Chemostat-Based Biofilm Fermenter (CBBF) for the development of mature Enterococcus faecalis ATCC 29212 biofilms. The biofilms were grown for three days in Brain Heart Infusion (BHI) medium at pH 7.0 and 37 °C. The fermenter was first inoculated with an overnight culture of E. faecalis and then continuously supplied with fresh medium (half-strength BHI supplemented with 20% glucose w/v and 40 mM phosphate-citrate buffer) at a rate of 1.6 mL/min, simulating the dilution rate of human saliva [12, 20, 22]. Several control samples were prepared to establish baseline parameters for Confocal Laser Scanning Microscopy (CLSM) imaging: (1) specimens with no aging and CBBF incubation without bacterial inoculation or staining, (2) specimens with no aging but inoculated and stained after CBBF incubation, and (3) unaged, unstained, and non-incubated specimens.

Outcome assessment

Upon completion of incubation, the samples were aseptically removed from the fermenter, rinsed gently with sterile distilled water, and treated with the LIVE/DEAD® BacLight™ Bacterial Viability Stain (Invitrogen, Waltham, MA, USA). Each specimen was examined under a confocal laser scanning microscope (Zeiss LSM710, Carl Zeiss Canada Ltd., Toronto, ON, Canada) to evaluate interfacial morphology, bacterial proliferation, penetration depth, and cell viability. The biomaterial–dentin interface was localized, and three predefined regions of interest were imaged per specimen using a 20× water-immersion objective (NA 1.0), yielding a total of nine regions per experimental group. Z-stack images were captured at 1 µm intervals and analyzed using IMARIS software (Bitplane AG, Zürich, Switzerland) [23].

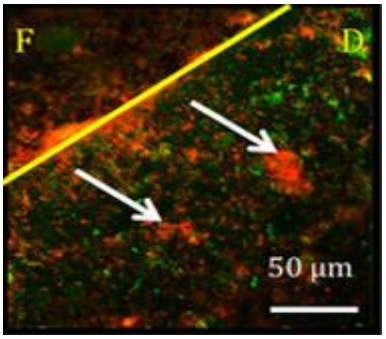
Statistical analysis

Data were tested for normality (Shapiro–Wilk) and homogeneity of variances (residual plots) before applying a three-way ANOVA to determine the effects of biomaterial type, aging medium, and time on biofilm penetration depth and bacterial viability within the biomaterial–dentin interface. Post hoc comparisons were conducted using Scheffé's test, with statistical significance set at $p < 0.05$.

Results and Discussion

Bacterial biofilm proliferation depth

Biofilm infiltration along the biomaterial–dentin interface was evident in all experimental groups, as exemplified by the representative AH group specimen shown in **Figure 1**. The 30-day BC group could not be evaluated due to extensive material expansion that obscured visualization of the interfacial boundary.



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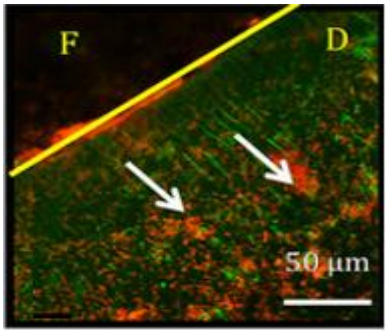

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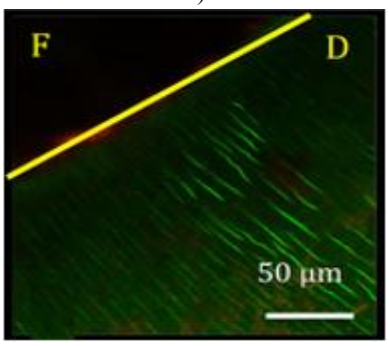
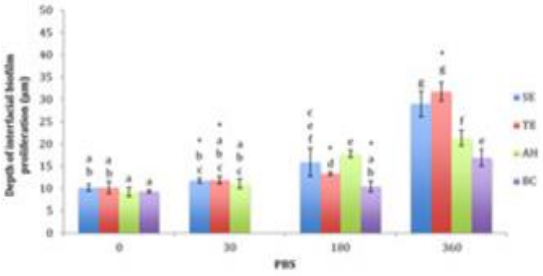


c)

Figure 1. Confocal Z-stack images of a specimen from group AH, aged for thirty days in PBS, processed using IMARIS. The yellow line indicates the interface between the root canal filling (F) and dentin (D) at depths of 0 µm (a) and 5 micrometers (b). White arrows point to bacterial biofilm. No biofilms were observed at 13 micrometers (c). Using the Live/Dead kit, green fluorescence represents live cells, and red indicates dead cells.

Figure 2 shows the depths of biofilm growth for the different groups, aging durations, and media. In the unaged control samples, bacterial proliferation at the interface was comparable across all four groups ($p > 0.05$). After 360 days, biofilm extended significantly deeper in every group, irrespective of aging in PBS or SHSE ($p < 0.05$). The TE and SE groups consistently exhibited the most extensive biofilm penetration, while AH and BC had shallower proliferation, with differences influenced by the type of aging medium.



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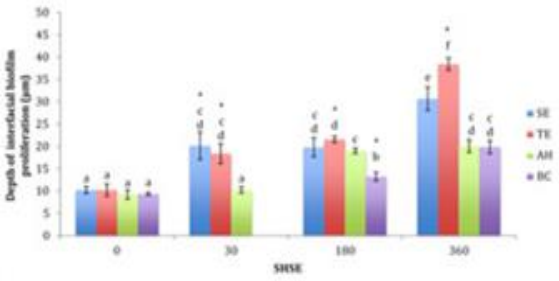

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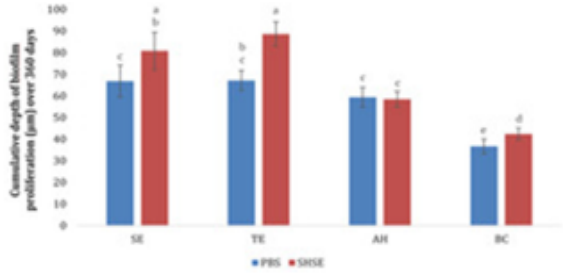
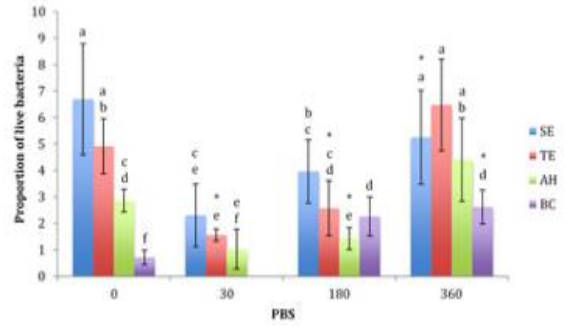


c)

Figure 2. The depth of biofilm growth at the material–dentin interface (µm) is shown for SE (self-etch adhesive), TE (total-etch adhesive), AH (epoxy-resin sealer), and BC (bioceramic sealer) before aging and after 30, 180, and 360 days in (a) PBS or (b) SHSE. Panel (c) summarizes the maximum biofilm penetration for each material over all aging periods in both media. Values are expressed as mean ± SD. The BC group displayed the shallowest bacterial penetration. Different letters indicate statistically significant differences among materials at each aging time within the same medium, while * highlights significant differences between PBS and SHSE for the same material at the same time point. SE and TE showed notable increases in biofilm depth after 30 days in SHSE ($p < 0.001$) and 180 days in PBS ($p < 0.05$). AH experienced significant deepening after 180 days in both media ($p < 0.05$). Across all aging periods, SHSE generally promoted greater biofilm penetration than PBS in SE, TE, and BC, whereas AH's biofilm depth was not significantly affected by the incubation medium.

Live bacteria proportion

Figure 3 illustrates the percentage of live bacteria across the different materials, aging durations, and media. Initially, BC contained significantly fewer live bacteria than AH, TE, and SE ($p < 0.05$). All groups showed a marked decrease in live bacterial proportion after 30 days of aging in either PBS or SHSE, followed by a significant rebound at 180 days in both media ($p < 0.05$). SHSE favored higher proportions of live bacteria for TE at 30 and 180 days, AH at 180 days, SE at 360 days, and BC at 360 days ($p < 0.05$). By 360 days, BC consistently exhibited the lowest proportion of live bacteria regardless of the aging medium ($p < 0.05$).



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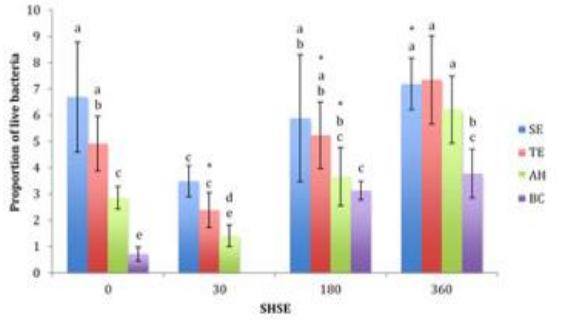

b)

Figure 3. Proportion of live bacteria within interfacial biofilms is shown for the test groups—SE (self-etch adhesive), TE (total-etch adhesive), AH (epoxy-resin sealer), and BC (bioceramic sealer)—before and after 30, 180, or 360 days of aging in (A) PBS or (B) SHSE. Data are presented as mean ± SD. * Marks significant differences between PBS and SHSE for the same material at the same time point.

This study evaluated the growth of E. faecalis biofilms at the junction between root dentin and four commonly used endodontic materials to assess the durability of the material–dentin interface. Bacterial infiltration occurs when small gaps or voids develop at this interface, indicating incomplete sealing or degradation over time. None of the materials tested provided an initially bacteria-proof seal. With prolonged aging, biofilm growth increased, suggesting that interfacial breakdown allows deeper bacterial penetration. The observed biofilm depth and cell viability varied depending on the type of material, aging medium, and duration, supporting the hypothesis that these factors influence interfacial stability.

Specimens were aged individually in sterile vials containing PBS or SHSE for up to 360 days. After this period, they were placed in a continuous bacterial flow system with BHI medium to promote E. faecalis biofilm formation under controlled, pathogen-simulating conditions. E. faecalis, a facultative anaerobe, was selected as a model organism because it is a representative endodontic pathogen that is easy to culture and provides a reliable marker for interface integrity, consistent with previous research [3, 4, 11, 12, 15, 16, 18, 20, 24]. However, these in vitro results may not fully reflect clinical conditions, and multi-species biofilm models may be necessary to evaluate the true pathological relevance of increased bacterial penetration due to material degradation. The staged aging in PBS or SHSE was implemented to avoid interactions between SHSE proteins and bacteria, isolating the effects of aging while accommodating the practical limits of long-term biofilm cultivation [12, 20].

SHSE was used to mimic enzymatic activity from saliva, blood, bacteria, and neutrophils in the oral environment. It accelerated interfacial breakdown in BC, TE, and SE materials and generally increased the proportion of live bacteria across all groups at specific time points. The esterase components in SHSE (CE and PCE) produce hydrolytic activity similar to that in the oral cavity, which can promote degradation of methacrylate-based composites and adhesives, weaken bonding to dentin [18], increase biofilm proliferation at the interface [12], reduce microhardness, cause weight loss, and lead to dimensional changes. These effects may collectively influence the clinical performance of resin adhesives and bioceramic sealers [16]. The findings emphasize the importance of simulating oral enzymatic conditions when testing endodontic biomaterials in vitro.

Confocal laser scanning microscopy (CLSM) is a non-invasive technique that allows the in situ examination of biofilms, avoiding the limitations of traditional two-dimensional destructive methods, such as scanning electron microscopy [12, 25]. CLSM enables detailed visualization of the biofilm's three-dimensional architecture and the spatial distribution of specific bacteria [26]. However, its application to clinically obtained samples can be challenging because confocal imaging has a limited penetration depth, requires

parallel canal walls for accurate analysis, and clinical canals are typically tapered. Additionally, the opacity of one of the tested sealers (BC) restricted imaging depth across all material groups in this study.

The use of multiple fluorescent stains allows quantification of bacterial cells within biofilms and assessment of their viability [25], providing an indication of the antimicrobial properties or cytotoxicity of the tested materials [27]. In this study, stained bacteria were used to determine whether the conditions within the biomaterial–dentin interface supported the formation of live pathogenic biofilms.

All tested materials initially provided comparable sealing ability and resistance to bacterial penetration, but this performance declined following exposure to both PBS and SHSE. Among the materials, BC aged in PBS for 360 days exhibited the least interfacial bacterial proliferation, suggesting a relatively stable material–dentin interface, possibly aided by the sealer's prolonged alkaline pH and release of bioactive glass [28]. Previous studies have shown that exposure to SHSE can reduce BC's setting expansion and increase solubility [16]. In the current work, imaging the 30-day-aged BC specimens was challenging, likely due to initial setting expansion obstructing the CLSM field; subsequent aging and potential material dissolution allowed clearer imaging.

AH has been reported to be more resistant to hydrolytic degradation than other sealers [16], a finding confirmed in this study, as AH showed minimal effects from SHSE exposure. This resistance may be attributed to its hydrophobic nature and absence of ester bonds susceptible to hydrolysis [29].

In contrast, the methacrylate resin-based TE and SE adhesives were more prone to interfacial biofilm formation. Incubation in SHSE further increased this susceptibility, likely due to hydrolysis of ester linkages within the resin. Additionally, post-polymerization shrinkage of these materials may contribute to interfacial gaps, compromised sealing, and increased biofilm proliferation observed in this study [16]. Despite being more hydrophilic and theoretically more vulnerable to hydrolytic degradation, SE exhibited less bacterial penetration than TE after 360 days in SHSE. This observation aligns with findings by Serkies *et al*. [18], who reported superior interfacial integrity of SE versus TE under similar conditions. SE's better performance may result from its simplified application in complex root canal geometries, as it does not require a separate etch-and-rinse step, reducing the risk of procedural error [30], and from its inherent chemical composition, which may confer greater enzymatic resistance [24]. Although phosphoric acid etching in TE systems may activate endogenous matrix metalloproteinases in dentin, our experience suggests this effect is minor compared with the collagenolytic activity of E. faecalis and unlikely to significantly affect TE performance [4, 18]. The poorer performance of restorative adhesives as root canal sealers, noted here, is consistent with previous studies [8]; nevertheless, including these materials serves as a positive control to highlight susceptibility to interface degradation and clarifies the reasons behind their inferior sealing performance in endodontics.

The observed increase in live bacterial proportions in certain SHSE-aged groups may result from interactions between SHSE proteins and bacteria, as well as from the enlargement of interfacial gaps caused by SHSE-mediated degradation of the material over time [12, 31]. Across all groups, BC consistently displayed the lowest proportion of live bacteria, suggesting intrinsic antimicrobial properties, potentially related to its alkaline pH. Antimicrobial activity in AH and BC declined over time, consistent with previous reports [32, 33]. SE, TE, and AH have been shown to exhibit initial antimicrobial effects immediately after setting [13]. However, in the present study, all materials showed a reduction in live bacteria after 30 days of pre-incubation compared to baseline. Although the highest cytotoxic or antimicrobial effects would theoretically occur immediately post-placement due to initial compound release, the data suggest a delayed effect. This may be attributable to the slow initial diffusion of cytotoxic compounds from the sealer, delayed generation of degradation by-products in SHSE, gradual movement of compounds from the interface toward the surrounding environment, and possible accumulation within the interface over time. Consequently, the concentration of cytotoxic agents may reach antimicrobial levels closer to the 30-day mark. Later, between 30 and 180 days, diffusion dynamics may shift, reducing cytotoxic compound concentration at the interface and diminishing antimicrobial effects. These findings indicate that assessing interfacial or eluent cytotoxicity over time provides a more meaningful evaluation of endodontic biomaterial performance than immediate cytotoxicity testing alone, as latent effects emerge only after accumulation of sealer by-products.

While this study successfully highlights the role of biodegradative factors in endodontics, further research is needed to explore the pathological consequences of these interactions. Limitations of the current work include the use of a monospecies biofilm, static thermomechanical incubation, and a simulated enzymatic environment. Future studies could employ

multispecies biofilms or in situ models, such as appliance-bound specimens in human mouths, to better replicate clinical conditions. Non-destructive techniques should continue to be used to evaluate biofilm penetration and potential damage to biomaterials or host tissues, helping to overcome CLSM limitations. Conducting fully clinical studies remains difficult, as assessing chemical degradation and interfacial biofilm properties without disrupting endodontic treatment is extremely challenging.

This investigation also examined a limited set of parameters for endodontic treatment. While the interplay between material chemistry and biodegradative factors is clearly important for long-term performance, future research should incorporate additional treatment strategies, such as antimicrobial agents, remineralization compounds, or biofilm-modulating interventions. Testing these approaches alongside biodegradative factors will provide a more complete understanding of material performance and treatment efficacy.

Conclusion

The findings of this study demonstrate that interactions between biodegradative media and root canal sealers significantly influence pathogen markers and should be considered in both material development and clinical practice. Exposure to media mimicking the hydrolytic activity of saliva, blood, bacteria, and immune cells affected interfacial biofilm proliferation, depth, and the proportion of live bacteria, emphasizing the importance of using aging media that reflect oral enzymatic activity when evaluating endodontic biomaterials. Moving forward, designers of endodontic materials should prioritize hydrolytic stability even in the absence of bacterial challenge, while clinicians should consider not only handling and immediate sealing performance but also the long-term interactions and degradation potential of these materials within the patient's oral environment.

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Ethics Statement: None

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