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Cytotoxic and Antibiofilm Properties of Antibiotic-Loaded Thermoresponsive Hydrogels for Root Canal Therapy

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Abstract: Dental infections can disrupt root development in immature permanent teeth, making traditional endodontic treatment challenging. Apexogenesis, a regenerative approach that promotes natural root development, offers a potential solution. However, issues related to disinfection and material biocompatibility still remain. The objective of this study was to evaluate the synergistic antimicrobial and antibiofilm properties of double and triple antibiotic combinations against common oral pathogens, and to incorporate the most effective combination into a thermosensitive hydrogel, to develop an alternative intracanal medication. Antibiotics were tested alone and in combination in planktonic and biofilm conditions of oral bacteria and *Candida albicans*. The antibiotic combinations with potential antimicrobial synergy were tested on *Enterococcus faecalis* biofilms in radicular dentin by confocal microscopy. Metronidazole (ME), ciprofloxacin (CI), and fosfomycin (FO) were incorporated into poly(N-vinylcaprolactam) (PNVCL) hydrogels, and their antibiofilm activity was compared to PNVCL hydrogels containing chlorhexidine (CHX) or calcium hydroxide (CH). The cytotoxicity of the hydrogels was assessed on MDPC-23 odontoblast-like cells using metiltetrazolium assays. A statistical analysis was performed using ANOVA followed by Tukey's test ($p < 0.05$). The combination of ME + CI + FO showed superior antibiofilm effects in mono- and dual-species biofilms and on biofilms inside dentinal tubules, comparable to CHX. PNVCL hydrogels with ME + CI + FO significantly reduced *E. faecalis* biofilms in dentinal tubules, exhibiting a higher efficacy than PNVCL + CH. Cytotoxicity tests revealed minimal effects on cell viability for both PNVCL hydrogels with and without antibiotics. In conclusion, ME + CI + FO showed potent antimicrobial synergy and, when loaded in thermosensitive PNVCL hydrogel, demonstrated significant antibiofilm activity and low cytotoxicity. These findings emphasize the potential of this formulation as an effective and biocompatible endodontic medication, especially for the treatment of immature permanent teeth.

Keywords: antibiotics; cytotoxicity; cell culture; hydrogel; chlorhexidine; calcium hydroxide

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1. Introduction

Dental infections can result in irreversible pulp damage, interrupting root development and the closure of the root apex in immature permanent teeth [1]. The large root canals and thin dentin walls make it difficult to perform conventional endodontic procedures, often resulting in the extrusion of irrigants and medicaments past the apical foramen [2,3]. In addition, even in mature teeth, studies have demonstrated that chemical–mechanical preparation alone is insufficient for achieving the complete disinfection of the root canal systems [4,5]. The analysis of microbial data from culture and molecular studies has appointed the presence of Gram-positive bacteria, Gram-negative bacteria, and fungi at the end of the endodontic treatment or between treatment sessions [6,7]. However, among these persistent microorganisms, Gram-positive facultative bacteria such as *Streptococcus*, including *S. mutans*, *Actinomyces*, including *A. israelii*, *Enterococcus faecalis* [6,7], and *Candida* spp., especially *C. albicans* [8], are more frequently identified. These microorganisms are particularly problematic in endodontic infections due to their ability to form biofilms on dentin surfaces and resistance to conventional antimicrobial treatments [5,8,9]. *Enterococcus faecalis*, for example, is a common pathogen in endodontic infections, notorious for its resistance to root canal irrigants and its ability to survive in harsh environments [9]. *Candida* spp., often coexisting with bacterial pathogens, further complicates treatment by contributing to mixed infections that are more challenging to eradicate [5,8].

The conventional treatment for managing immature teeth with pulp or periapical conditions focuses on stimulating the formation of a hard tissue barrier at the root apex to facilitate apical closure, a process known as apexification [2,10]. Apexification is a procedure aimed at removing the coronal necrotic and potentially infected pulp, with the goal of preserving the vitality of the root apex and promoting continued root development [2,10]. However, this approach can disrupt root development, potentially affecting the root length and the structural integrity of the dentin and increasing the risk of fractures [11]. Clinical trials have shown that apexogenesis, a regenerative approach aimed at promoting natural root development, is achievable for both vital and non-vital pulp in immature teeth [12–18]. Apexogenesis relies on the activation of remaining dentin pulp stem cells or the recruitment of new cells from the periapex, leading to the formation of a functional apical barrier and continued root development [1,11]. This method not only prevents the interruption of natural root growth but also results in roots with improved structural integrity [1,2]. Most apexogenesis protocols involve passive irrigation with sodium hypochlorite at low concentrations, combined with intracanal medication using triantibiotic paste (TAP) containing ciprofloxacin, metronidazole, and minocycline [12–18]. These antibiotics are chosen for their broad-spectrum activity against the microbes commonly found in root canal infections, such as *E. faecalis* and *C. albicans* [19–22]. However, the use of minocycline in TAP raises concerns due to potential tooth discoloration and cytotoxicity, highlighting the need for safer alternatives in the treatment of immature teeth [13,21].

New alternatives to minocycline have been studied for the paste, such as fosfomycin, a potent inhibitor of MurA, an enzyme critical for the biosynthesis of peptidoglycan, which is essential for bacterial cell wall formation. Fosfomycin exerts its antimicrobial effects by interfering with the initial stage of peptidoglycan synthesis, thus preventing bacterial cell wall assembly and leading to bacterial lysis. This broad-spectrum antibiotic is effective against both Gram-positive and Gram-negative bacteria and fungi, due to its ability to inhibit MurA, which is conserved across bacterial species [20–22]. Another issue relates to the high concentration of each TAP component (1 g/mL), which has been linked to toxic effects on undifferentiated cells, crucial for the regenerative process [23]. Recent studies, however, have tested these antibiotics at lower concentrations, preserving their antimicrobial efficacy while minimizing adverse effects on surrounding cells [24,25].

Polymers have been employed in therapeutic delivery systems to develop sustained-release formulations for advancements in endodontic treatments, maintaining bacterial activity for a long period of time, which reduces the possibility of bacterial resistance, as it avoids the peak-and-trough phenomenon associated with conventional antibiotic delivery methods, where the drug concentration fluctuates and may fall below the therapeutic threshold, allowing for bacterial adaptation [26,27]. The main polymeric carriers are currently being used in different forms: scaffolds, hydrogels, and nanoparticles, or combinations of these forms [28]. Hydrogels are able to transition from the liquid to the gel (sol–gel) by the formation of a polymeric network, because of physical interactions.

Thermoresponsive hydrogels have emerged as a promising alternative due to their ability to transition from liquid to gel at body temperature, facilitating ease of application and removal without the need for additional instruments [26]. Smart thermosensitive hydrogels, such as poly N-vinylcaprolactam (PNVCL), exhibit lower critical solution temperatures (LCST) that allow them to transition to gel form at human physiological body temperature. This transition occurs due to changes in hydrogen bonding and Van der Waals interactions between the polymer chains and solvent molecules [28]. In addition, PNVCL-based hydrogels are advantageous because they do not hydrolyze to produce toxic by-products and have demonstrated excellent cytocompatibility [29]. When exposed to temperatures lower than the human body temperature, these hydrogels revert to a liquid state, enabling them to be removed from the injection site if necessary [30]. These hydrogels also allow for the controlled release of therapeutic agents, reducing toxicity and enhancing treatment precision [29,30]. However, while existing studies have explored various apexification protocols, there is a notable gap in the application of thermoresponsive hydrogels in this context. Thus, this study aimed to evaluate the antimicrobial and antibiofilm properties of several antibiotic combinations against potential oral pathogens, as well as to evaluate the cytotoxicity and antibiofilm effectiveness of a PNVCL hydrogel containing the most effective antibiotic combination, aiming to identify alternative medicaments for the endodontic therapy of young permanent teeth.

2. Material and Methods

2.1. Antibiotics and Controls Agents

All reagents, antibiotics, and salts were sourced from Sigma-Aldrich (St. Louis, MO, USA), while the culture media were obtained from Difco Laboratories (Kansas City, MO, USA). The antibiotics evaluated in this study included metronidazole (ME) (CAS No.: 443-48-1, Sigma-Aldrich), ciprofloxacin (CI) (CAS No.: 85721-33-1, Sigma-Aldrich), minocycline (MI) (CAS No.: 13614-98-7, Sigma-Aldrich), doxycycline (DO) (CAS No.: 24390-14-5, Sigma-Aldrich), and fosfomycin (FO) (CAS No.: 26016-98-8, Sigma-Aldrich) [17,20]. The antibiotics were individually dissolved in sterile deionized water at a concentration of 4 mg/mL. The controls, chlorhexidine digluconate (CHX) and amphotericin (APT), were dissolved in water at concentrations of 20 mg/mL and 4 mg/mL, respectively. All the solutions were filtered using 0.2 μm syringes and stored appropriately. Table 1 presents an overview of the antibiotics, control agents, and antibiotic combinations used in this study.

Table 1. Antibiotics, controls, and antibiotic combinations used in this study.

Antibiotics	Double Combinations	Triple Combinations
Metronidazol (ME)	ME + CI	ME + CI + MI
Ciprofloxacin (CI)	ME + MI	ME + CI + FO
Fosfomycin (FO)	ME + DO	ME + MI + FO
Doxycycline (DO)	ME + FO	ME + CI + DO
Minocycline (MI)	CI + MI	ME + DO + FO
Chlorhexidine (CHX)	CI + DO	CI + DO + FO
Amphotericin (APT)	CI + FO	CI + MI + FO
	DO + FO	
	MI + FO	

2.2. Antimicrobial Activity Assays

2.2.1. Cultivation and Conditions of Microbial Strains

The reference strains used in this study were *Enterococcus faecalis* (*E. faecalis*, ATCC 51299), *Streptococcus mutans* (*S. mutans*, ATCC 25175), *Actinomyces israelii* (*A. israelii*, ATCC 12102), and *Candida albicans* (*C. albicans*, ATCC 26790). Strains suspensions were cultured on Mitis Salivarius Agar (Difco Laboratories, Kansas City, MO, USA) supplemented with 0.2 U/mL bacitracin (Sigma-Aldrich) for *Streptococcus mutans*, Brain Heart Infusion Agar (BHIA, Difco Laboratories) for *A. israelii* and *E. faecalis,* and incubated at 37 °C for 24 h in a 5% CO_2 atmosphere. *C. albicans* was cultivated in Sabouraud Dextrose Agar (SDA) containing 40 mg/mL chloramphenicol at 37 °C in aerobic conditions for 24 h. Bacterial cultures were adjusted to 1–5 $\times 10^8$ CFU/mL and *C. albicans* cultures to 1–5 $\times 10^6$ CFU/mL, using spectrophotometry and counting by agar plating [31]. All the experiments were conducted in triplicate across three separate trials (n = 9/group).

2.2.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bacterial/Fungicidal Concentration (MBC/MFC)

The MIC and MBC/MFC were determined using the microdilution method with 96-well microtiter plates, based on the criteria of the Clinical Laboratory Standards Institute (CLSI) for bacteria (CLSI, M27-A2) and for yeasts (CLSI, M7-A5) [32,33]. The antimicrobial agents (antibiotics, CHX, and APT) were serially diluted to create concentrations between 0.24 µg/mL and 2000 µg/mL. Suspensions of microorganisms, previously adjusted in Miller–Hinton (MH) broth for bacteria or RPMI for *C. albicans*, were added to each well with the respective dilutions. The microplates were placed in an incubator at 37 °C for 24 h. Following this, 0.01% resazurin was added to each well, and incubation continued for an additional 4 h to assess cell viability. Aliquots from the blue-stained wells were serially diluted and plated onto MH Agar for bacteria and Sabouraud Dextrose Agar (SDA) for *C. albicans* and incubated for 48 h. After incubation, colony-forming units (CFU/mL) were counted. Minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) were established when the agents eliminated over 99% of the microorganisms.

2.2.3. Effect of Antibiotic Combinations on Microorganisms in Planktonic Conditions

The antibiotics were used in double or triple combinations at their respective MIC values and incubated with the microbial cultures under the same conditions as described earlier for 24 h. After incubation, the wells were treated with resazurin, and samples from the stained wells were then diluted, plated, and incubated at 37 °C for 48 h. CFU/mL counts were then determined [31]. The antibiotic combinations with the best results were selected for all subsequent experiments, with the concentration set at 5× the MBC against *E. faecalis*, except for ME, which was considered at 312.5 µg/mL.

2.3. Antibiofilm Activity Assays

2.3.1. Evaluation of Mono- and Dual-Species Biofilms on the Surface of Radicular Dentin Blocks

This research was approved by the Animal Experimentation Ethics Committee of the Araçatuba Dental School—UNESP (CEUA), under protocol number 2014/00595. Bovine roots were sectioned transversally with a precision cutter using two diamond discs (IsoMet 1000, Buehler, Lake Bluff, IL, USA) under water irrigation. The root dentin slices were cut into four blocks (3 × 3 × 0.7 mm) and sequentially polished with water-cooled silicon carbide paper discs (Extec Corp., Enfield, CT, IL, USA) on a polisher (BETA polisher, Buehler, Lake Bluff, IL, USA). The dentin blocks were subsequently cleaned using ultrasonic waves, first with 17% EDTA for 3 min and then with deionized water for 5 min. They were then autoclaved for 15 min at 121 °C and stored at 4 °C for later use [34,35].

Mono-species biofilms of *E. faecalis* and dual-species biofilms of *E. faecalis* and *C. albicans* were formed on the surface of dentin blocks to evaluate the antibiofilm activity of the most effective antibiotic combinations from previous assays. The dentin blocks were fixed to the bottom of 96-well microplates using sterile double-sided tape and inoculated with 100 µL of bacterial suspension (or 50 µL for each pathogen in dual-species biofilms) at a concentration of 1–5 × 10^7 CFU/mL in BHI with 0.5% glucose. The blocks were then randomly allocated into two treatment groups: (1) *Initial biofilm*—the antibiotic combination (at 5× MBC) was added 1 h after microbial adhesion to the dentin blocks; (2) *Mature biofilm*—the antibiotic combination (at 5× MBC) was applied to 48 h old biofilms that had already formed on the dentin blocks. After incubation at 37 °C in 5% CO_2, the dentin blocks were washed once with 100 µL of 0.9% saline and inserted into microtubes containing 500 µL of Cystein–Peptone composed of 5 g yeast extract, 1 g peptone, 8.5 g NaCl, 0.5 g L-cysteine HCl, and 100 mL of glycerine per liter, pH 7.3) and incubated at 37 °C for 1 h. Finally, the blocks were transferred to microtubes with deionized water and subjected to an ultrasonic bath for 20 min, then agitated using a vortex for 2 min. Microbial suspensions from each microtube were progressively diluted and cultured on M-Enterococcus Agar (Difco) for *E. faecalis* and SDA with chloramphenicol for *C. albicans*. After incubating the plates for 48 h, the CFU/mL was counted [34,35].

2.3.2. Effect of Antibiotics on *E. faecalis* Biofilms in Dentinal Tubules and Analysis by Confocal Laser Scanning Microscopy (CLSM)

Biofilm assays were conducted in dentinal tubules from bovine root canals for CLSM analysis, following the protocol outlined by Duque et al. [22], with slight alterations. Dentin blocks (4 mm in length) were extracted from bovine roots, and their canals were enlarged with a Gates Glidden drill#6 (Dentsply-Sirona, York, PA, USA) at low speed. Each cylindrical dentin block was then divided into two semi-cylindrical sections and refined with a fine carbide bur until reaching the final dimensions of 3 × 3 × 2 mm. The blocks (n = 6/groups) were cleaned and sterilized as previously described, then placed in a microtube with the canal side facing upwards and secured with resin composite. *E. faecalis* suspension at 10^7 CFU/mL in BHI broth was introduced into each microtube and harvested twice, each for 5 min. The dentin blocks were then incubated individually in BHI broth with 0.5% glucose for 14 days, replacing the culture medium every 48 h. After this period, the blocks were washed and exposed to antibiotic combinations (at 5× MBC) for 24 h in a new plate. Subsequently, the specimens were washed twice, sectioned into 1mm thick transverse slices, and stained with 100 µL of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR, USA). Two uninfected specimens were also stained as negative controls. Fluorescence was observed using CLSM (Leica TCS SP5, Microsystems GmbH, Mannheim, Germany), and images were captured with Leica

Application Suite-Advanced Fluorescence program (LAS AF, Leica). The quantification of the red fluorescence ratio in relation to green-and-red fluorescence was determined using COMSTAT 2 software, indicating the proportion of dead cells/total cells for each group [22].

2.4. Synthesis and Characterization of PNVCL and Incorporation of Antibiotics

PNVCL was synthesized and characterized following the method detailed by Sala et al. [36]. Briefly, PNVCL was obtained by adding 15% of N-vinylcaprolactam monomer in DMSO and distilled water. Subsequently, 2% *w*/*w* of 2,2′-azobis (2-methylpropionitrile) was introduced into the reaction mixture, which was then heated to 70 °C under a nitrogen atmosphere. After polymerization, PNVCL was purified through dialysis against deionized water for 4 days. Following this, it was lyophilized and stored at 4 °C until use. To determine the lower critical solution temperature (LCST) of the polymer, the transmittance of a 1% *w*/*v* aqueous solution of PNVCL was measured at 500 nm using UV-Vis spectroscopy (MultiSpec-1501 UV-vis Spectrophotometer, Shimadzu, Kyoto, Japan) over a temperature range from 25 °C to 35 °C. Additionally, the particle size distribution, including the numerical values for the hydrodynamic diameter of PNVCL in a 1% *w/v* polymeric dispersion, was assessed. The antibiotics exhibiting the strongest antimicrobial effects (ME + CI + FO) were incorporated (at 0.625 mg/mL each) into the PNVCL hydrogel. PNVCL without antibiotics was considered as a control. CHX and CH were also added to the PNVCL hydrogel as positive controls.

2.5. Antibiofilm Activity of Hydrogels Containing Antibiotics in Radicular Dentin Tubules and CLSM Analysis

Semi-cylindrical dentin blocks from bovine roots were prepared as previously described [22] and infected with *E. faecalis* cultures, adjusted to 1–5 × 10^6 CFU/mL through sequential centrifugation. The microtubes were incubated at 37 °C in BHI medium supplemented with 0.5% glucose for 14 days, with a 5% CO_2 atmosphere. The culture medium was replaced every 48 h. Afterward, the dentin samples were taken out, rinsed, and exposed to the following solutions: (1) PNVCL without antimicrobials; (2) PNVCL loaded in a triantibiotic mixture (ME, CI, and FO at 0.625 mg/mL each); (3) PNVCL-loaded chlorhexidine (CHX) at 0.5 mg/mL; (4) PNVCL-loaded CH at 1 mg/mL; and (5) sterile water as a negative control. Each sample was placed in the designated solution for 48 h, initially stirred for 1 h, followed by a 1 min wash with sterile water at 37 °C. The samples were then sliced into two halves and analyzed longitudinally through the dentinal tubules using CLSM [22].

2.6. Cytotoxicity of Hydrogels Containing Antimicrobial Agents

Odontoblast-like cells (immortalized mouse dental papilla cells—MDPC-23) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics until they reached confluence, as previously described [37,38]. The cells were seeded at a density of 5 × 10^3 cells per well in a 96-well plate and pre-incubated at 37 °C for 24 h. Subsequently, they were exposed to serial dilutions (0.625 to 0.019 mg/mL) of antibiotics, controls (CHX and HC), and PNVCL hydrogel (diluted 8×), with or without the compounds (antibiotics at 0.625 mg/mL, CHX at 0.5 mg/mL, and CH at 1 mg/mL), and incubated for another 24 h. The supernatant was then removed, and cell viability was assessed using the methyltetrazolium (MTT) assay. Cell viability was assessed by measuring the absorbance at 570 nm using a microplate reader (Biotek, Winooski, VT, USA) [31]. The control group (DMEM) was used to establish 100% viability, and the cell viability of the test groups was expressed as a percentage relative to the control.

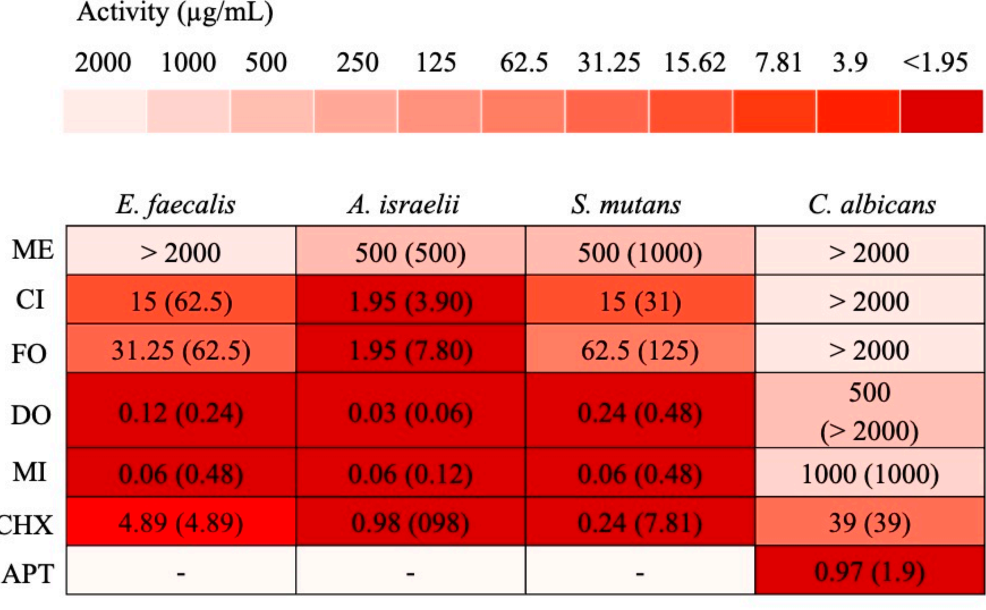
2.7. Statistical Analysis

Data analysis was conducted using SPSS version 17.0 (SPSS GmbH, Munich, Germany). The Kolmogorov–Smirnov test was employed to assess the normality of the data and homogeneity of variances. Following this, one-way ANOVA was used to analyze the variables, with Tukey's post hoc test applied for pairwise comparisons. A significance of $p < 0.05$ was considered for all the statistical tests.

3. Results

3.1. Antimicrobial Activity in Planktonic Conditions

In planktonic conditions, DO and MI exhibited the strongest antimicrobial effects against all the tested species, with MIC values ranging from 0.03 µg/mL to 0.24 µg/mL and MBC values from 0.06 µg/mL and 0.48 µg/mL. CI and FO showed activity against *S. mutans*, *A. israelii*, and *E. faecalis*, with MIC/MBC values ranging from 1.95 µg/mL to 62.5 µg/mL. ME exhibited antimicrobial effects on *A. israelii* and *S. mutans* at higher concentrations (at 500 µg/mL) but was ineffective against *E. faecalis*. As a positive control, CHX demonstrated efficacy against all the tested species. Tetracyclines at elevated concentrations significantly inhibited the growth *of C. albicans*, as well as by the controls amphotericin and CHX (Figure 1).



	E. faecalis	*A. israelii*	*S. mutans*	*C. albicans*
ME	> 2000	500 (500)	500 (1000)	> 2000
CI	15 (62.5)	1.95 (3.90)	15 (31)	> 2000
FO	31.25 (62.5)	1.95 (7.80)	62.5 (125)	> 2000
DO	0.12 (0.24)	0.03 (0.06)	0.24 (0.48)	500 (> 2000)
MI	0.06 (0.48)	0.06 (0.12)	0.06 (0.48)	1000 (1000)
CHX	4.89 (4.89)	0.98 (098)	0.24 (7.81)	39 (39)
APT	-	-	-	0.97 (1.9)

Figure 1. Heatmap of minimal inhibitory concentration (MIC) values for antibiotics and controls (CHX and APT) following 24 h of treatment, in planktonic conditions. Minimal bactericidal or fungicidal concentration (MBC/MFC) are presented in parentheses. Values are in µg/mL.

All the tested double antibiotic combinations significantly inhibited *A. israelii* growth (from −3.88 log for MI + FO to −10 log for CI + FO) in planktonic conditions, except by CI + DO and DO + FO (−1 log). All the combinations reduced *E. faecalis* growth (from −3.74 log for ME + FO to −7.6 log for ME + CI); however, it was eliminated only by CI + FO. Except by CI + MI (−4.21 log) and CI + DO (−5.77 log) which reduced bacterial counts, all the double antibiotic combinations eradicated *S. mutans* (Figure 2A). Among the triple combinations, ME + CI + FO significantly decreased *A. israelii* growth (−6.2 log) and completely eradicated *S. mutans* and *E. faecalis*. Similarly, the combinations ME + CI + MI and ME + MI + FO led to significant reductions in *A. israelii* (−8 log) and *E. faecalis* (−6 log), with ME + MI + FO also completely eradicating *S. mutans* (Figure 2B).

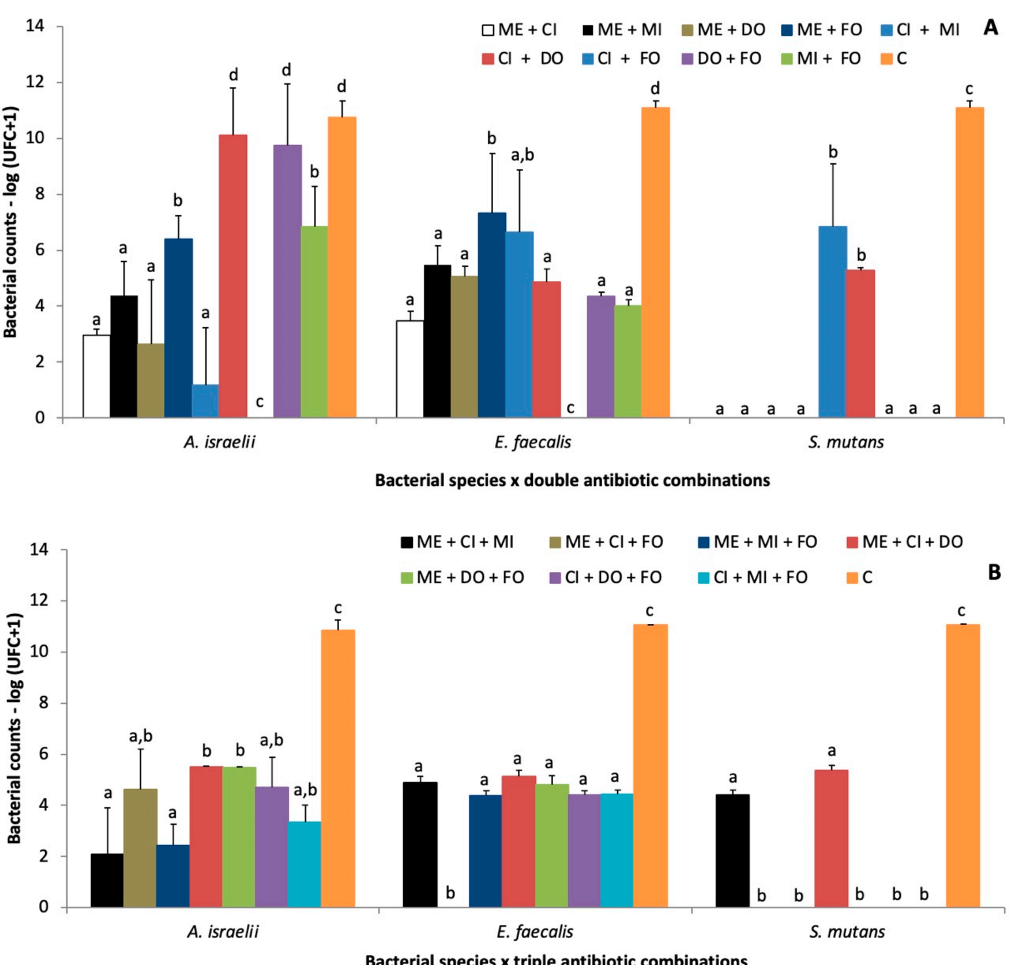


Figure 2. Antimicrobial activity of double (**A**) and triple (**B**) antibiotic combinations after 24 h of exposure under planktonic conditions. Control group (C) = culture medium with no antibiotics. Different lowercase letters indicate statistically significant differences among the antibiotic groups for each bacterial species, as determined by ANOVA and Tukey tests ($p < 0.05$).

3.2. Antibiofilm Effects of Antibiotic Combinations

In mono-species biofilms, all the triple combinations completely eliminated *E. faecalis* in the initial stages of biofilms and statistically reduced (−6 log) the mature biofilms (Figure 3A). In dual-species biofilms, ME + CI + FO and ME + MI + FO also eradicated *E. faecalis* in initial biofilms, and statistically diminished *E. faecalis* (from −2 to −2.5 log) in mature biofilms (Figure 3B). *C. albicans* counts were notably decreased by all the antibiotic combinations in both the initial (−3.6 log) and mature biofilms (from −1.52 to −2 log) (Figure 3C). CHX also significantly decreased the presence of *E. faecalis* and *C. albicans* in both the mono-species and dual-species biofilms (Figure 3A–C). According to CLSM results, ME + MI + FO and ME + CI + FO were the most effective combination against *E. faecalis* biofilms within the dentin tubules, reducing from 57% and 48% of cells, respectively, comparable to CHX (52.7%) (Figure 4).

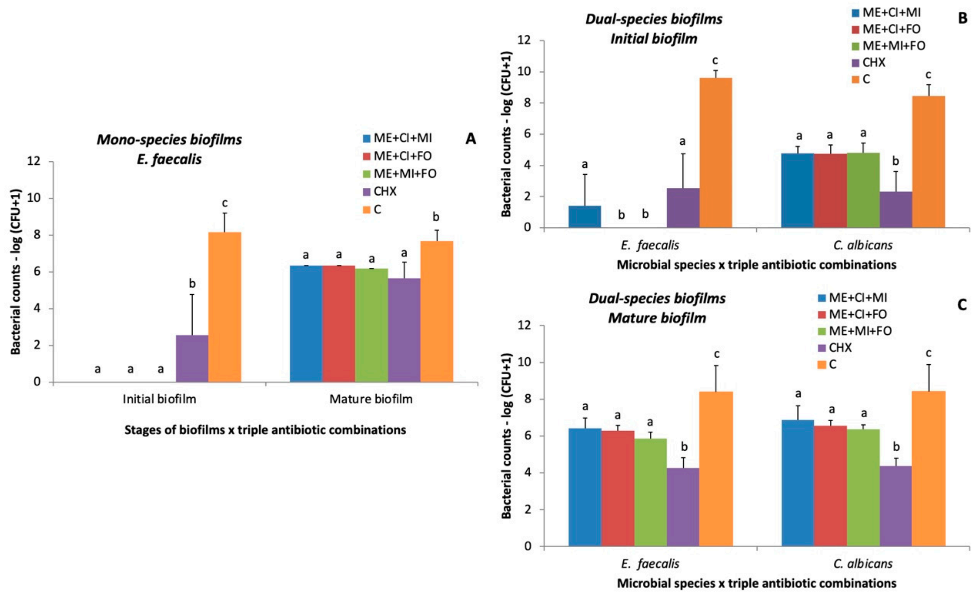


Figure 3. Effect of triple antibiotic combinations on initial and mature mono-species biofilms with *E. faecalis* (**A**), and on initial (**B**) and mature (**C**) dual-species biofilms with *E. faecalis and C. albicans* formed in dentin blocks. Control group (C) = culture medium with no antibiotics. Distinct lowercase letters indicate statistically significant differences among the antibiotic groups, taking into account each condition (initial/mature biofilms) or bacterial species separately, as determined by ANOVA and Tukey tests ($p < 0.05$).

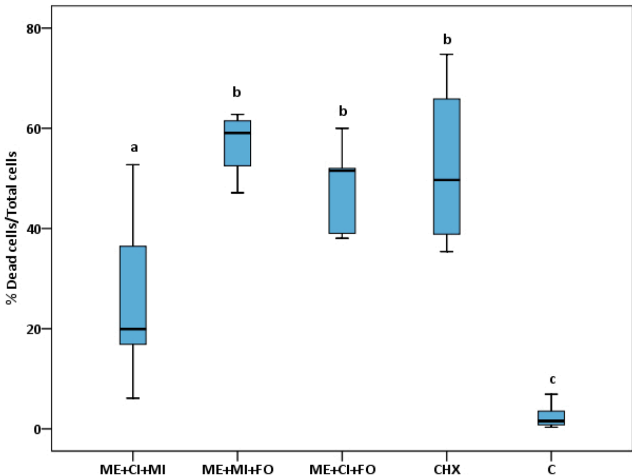


Figure 4. Effect of triple antibiotic combinations on *E. faecalis* biofilms formed within dentin tubules and analyzed using confocal microscopy. Control group (C) = culture medium with no antibiotics. Distinct lower-case letters indicate statistical differences among the groups, as determined by Kruskal–Wallis and Mann–Whitney tests ($p < 0.05$).

3.3. Evaluation of Polymeric Dispersion and Spectrophotometry of the PNVCL Hydrogel

The synthesized PNVCL hydrogel exhibited water solubility below its lower critical solution temperature (LCST) and became insoluble above this threshold (Figure 5A). The formation of PNVCL was confirmed using the liquid-state proton nuclear magnetic resonance (NMR), which also exhibited no residual monomers in the spectrum due to the dialysis process. The resulting hydrogel had an average molar mass (Mn) of 153,671 g/mol, a weight average molar mass (Mw) of 25,451 g/mol, and a polydispersity (Mw/Mn) of 6.04. The transmittance of PNVCL in water at a mass concentration of 1% was measured as a function of temperature, revealing an LCST of 33.5 °C. This value, which is below the

typical human body temperature, makes PNVCL suitable for applications in controlled drug delivery and transport (Figure 5B). Additionally, the hydrodynamic diameter (Dh) distribution of PNVCL at 25 °C and 37 °C was determined by dynamic light scattering (DLS). The distribution was narrow at the lower temperature, with an average Dh of 13 nm (Figure 5C). However, with the increase in temperature (Figure 5D), the Dh rapidly increases to an average value of 556 nm, confirming its sol-gel transition with the formation of a globular phase that can encapsulate the molecules of the different drugs.

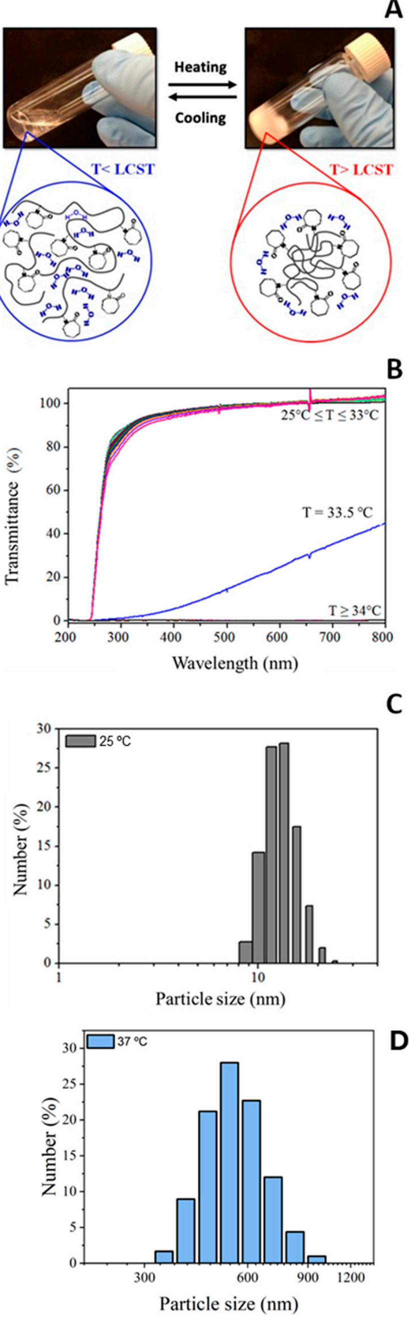


Figure 5. (**A**). Aqueous polymeric dispersion of PNVCL at room temperature (transparent and soluble) and when heated above the lower critical solution temperature (LCST), acquiring an opaque color due to phase separation and formation of polymeric aggregates. (**B**). UV-vis spectra at different temperatures of an aqueous polymeric dispersion containing 1% *w*/*v* PNVCL, synthesized at a solids content of 15%. (**C**). Numerical distribution of the hydrodynamic diameter of PNVCL in a polymeric dispersion with a concentration of 1% *w*/*v* at 25 °C and (**D**) at 37 °C.

3.4. Antibiofilm Activity of Hydrogels Containing Antibiotics by CLSM

The effects of PNVCL, PNVCL combined with antibiotics (PNVCL + ATB: ME—0.625 mg/mL, CI—0.625 mg/mL, FO—0.625 mg/mL), PNVCL combined with CHX (PNVCL + CHX: 0.5 mg/mL), and PNVCL combined with calcium hydroxide (PNVCL + CH: 1.0 mg/mL) were evaluated on *E. faecalis* biofilm formed in radicular dentin blocks. Representative confocal microscopy images illustrate the antibiofilm effects of PNVCL hydrogels containing antimicrobials on *E. faecalis* cells within dentin tubules. In these images, green fluorescence indicates live cells, while red fluorescence represents dead cells (Figure 6A–D). The PNVCL hydrogel alone exhibited minimal antibacterial effects, whereas PNVCL combined with antimicrobial agents enabled their release and demonstrated significant antibiofilm activity. Specifically, PNVCL + ATB achieved an 86.15% reduction in *E. faecalis* counts, similar to PNVCL + CHX, which resulted in an 85.38% reduction. In contrast, PNVCL + CH reduced viable cell counts by 56.31% (Figure 6E).

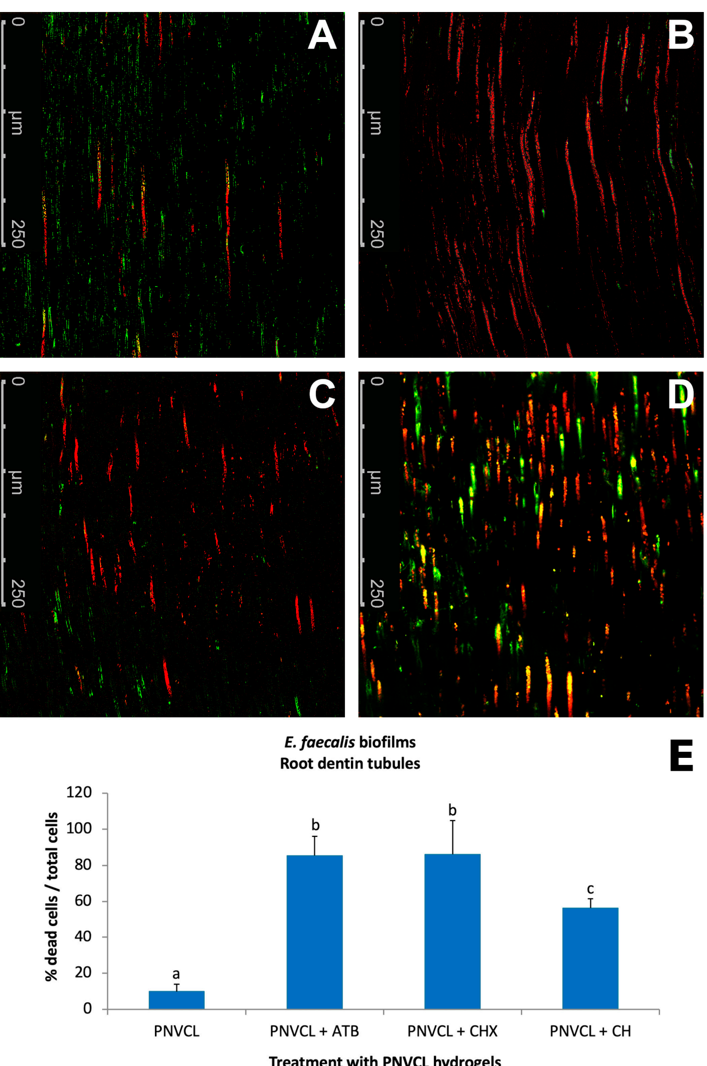


Figure 6. (**A**–**D**) Representative confocal microscopy images of *E. faecalis* biofilms inside dentin tubules after 48 h of the treatment with the following groups: (**A**). PNVCL; (**B**). PNVCL + ATB (ME + CI + FO combination); (**C**). PNVCL + CHX (chlorhexidine); and (**D**). PNVCL + CH (calcium hydroxide). In green: living cells. In red: dead cells. (**E**). Mean (SD) of the percentage of dead cells of *E. faecalis* obtained from biofilms formed for 14 days in radicular dentin tubules and exposed to the PNVCL hydrogels containing or not containing antibiotics (ATB), CHX, and CH for 48 h. Different lowercase letters indicate statistical differences among the groups, based on ANOVA and Tukey tests ($p < 0.05$).

3.5. Effect of Hydrogels Containing Antimicrobial Agents on Odontoblast-Like Cells Viability

The effects of exposing MDPC-23 cells to serial dilutions of antibiotics (ME, CI, or FO), CHX, and CH for 24 h are shown in Figure 7A. Antibiotics promoted a minimal reduction in cell metabolism, maintaining cell growth above 70%. CH was cytocompatible from 0.312 mg/mL and CHX, the most cytotoxic compound, from 0.039 mg/mL. PNVCL hydrogel was diluted 1/8 and it was not toxic to MDPC-23 cells [31]. Formulations containing PNVCL + ATB and PNVCL + CH maintained high cell viability, with growth above 80%. Conversely, PNVCL + CHX exhibited the most significant toxic effect on MDPC-23 cells, resulting in only 3.89% growth (Figure 7B).

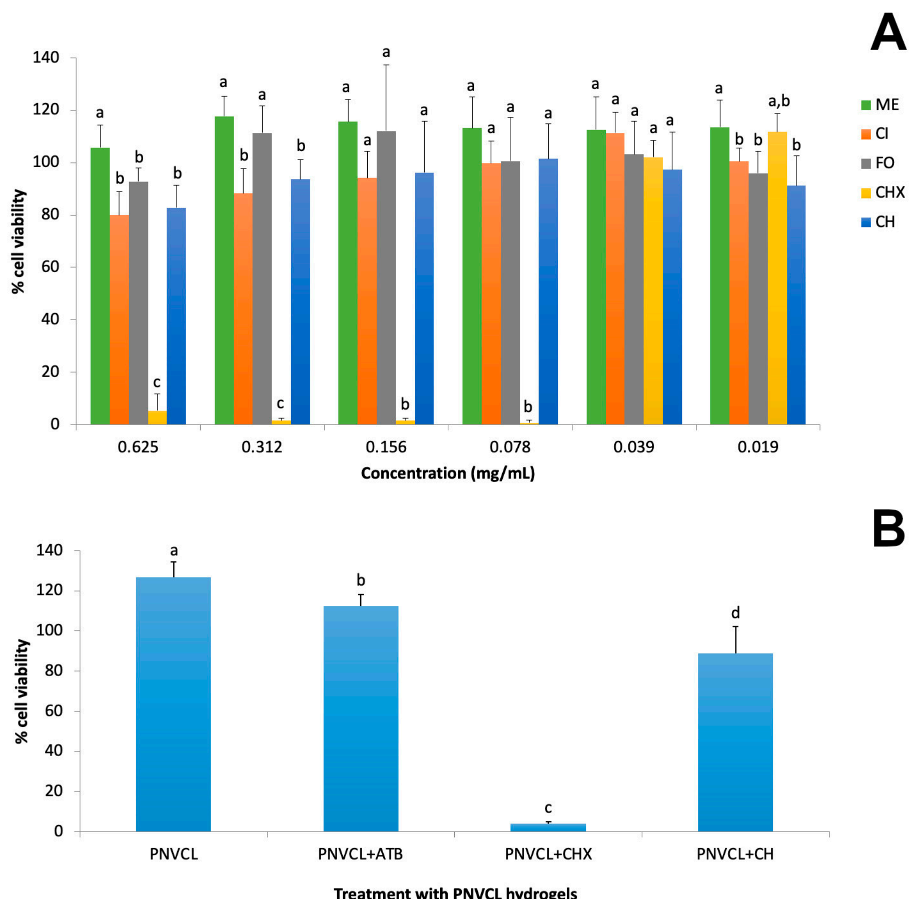


Figure 7. (**A**). Means (SD) of the percentage of viable MDPC-23 cells assessed following exposure to serial dilutions of antibiotics (ME, CI, or FO), CHX, and CH for 24 h. (**B**). Mean (SD) of the percentage of viable MDPC-23 cells assessed following exposure to PNVCL, PNVCL + ATB (ME + CI + FO combination), PNVCL + CHX, and PNVCL + CH for 24 h. Different lowercase letters indicate statistical differences among the groups, based on ANOVA and Tukey tests ($p < 0.05$).

4. Discussion

Systemic antibiotics are typically avoided in treating endodontic failures or perirradicular lesions. However, the local application of antibiotics in pastes or other release vehicles may be beneficial for eliminating residual bacteria and promoting pulp or periapical healing [11–22]. In this study, the selection of antimicrobial compounds was mainly guided by the research conducted by Hoshino et al. [19] and Trope [18]. In addition, ME and CI have been used in double combination or in triple combination with minocycline (MI) for endodontic therapy of immature permanent teeth, because of their strong efficacy against both Gram-positive and Gram-negative bacteria, since endodontic diseases are polymicrobial infections [17–22]. Considering that MI causes tooth discoloration and cytotoxity [13,21],

we included fosfomycin (FO) in the study as an alternative antibiotic to minocycline with a similar effect against bacterial strains (Gram-positive and Gram-negative) and with the ability to prevent bacterial biofilm development [20,21].

Our findings indicate that DO and MI exhibited the most potent bactericidal effect, while CI and FO showed a comparatively lower effectiveness against the tested species. These results align with previous studies, where DO demonstrates a strong antimicrobial activity, exhibiting an MIC between 0.06 and 0.25 μg/mL against various bacterial strains, including *A. israelii* [39,40]. However, the results for CI differ, as we observed an MIC90 > 32 μg/mL for *A. israelii*, which contrasts with other studies that reported lower MIC values for this antibiotic [39,40]. Regarding fosfomycin, our results were consistent with those of others, showing an MIC of 31.25 μg/mL against *E. faecalis* [41]. This supports the notion that FO is an effective antimicrobial agent for *E. faecalis* in root canal therapy. On the other hand, our study found a lower efficacy for CI, with MIC values notably higher than those reported by other researchers, such as 0.2 μg/mL for *E. faecalis* [42]. This difference could be attributed to variations in the experimental conditions, such as bacterial strains, culture media, or testing methods used. In contrast, ME in our study showed an inhibitory effect only at high concentrations, specifically against *A. israelii* and *S. mutans*. This finding agrees with other studies that have reported limited effectiveness of metronidazole against certain species of *Actinomyces*, with MIC values reaching as high as 256 μg/mL [40]. Furthermore, ME, CI, and FO exhibited bacteriostatic properties at lower concentrations. This could explain the higher concentration-based minimum bactericidal concentration (CBM) values observed in our study, which likely reflect the concentrations required to cause bacterial death.

In this current study, the combination of CI and FO effectively eliminated all three bacteria evaluated, followed by the combinations of ME + CI, ME + MI, and ME + DO. This observation aligns with previous findings, where FO demonstrated a synergistic effect when combined with other antibiotics, such as minocycline, ampicillin, rifampicin, linezolid, teicoplanin, tigecycline, and vancomycin, in 89% of vancomycin-resistant *E. faecalis* strains [43]. The synergistic activity of fosfomycin in combination with these antibiotics further supports its potential as an effective antimicrobial agent, particularly in challenging clinical scenarios, like resistant bacterial infections. Despite the double combination of CI + FO showing a strong efficacy against all the bacteria evaluated, triple combinations including ME were chosen for the subsequent biofilm assays, considering that ME has a broad bactericidal spectrum against strict anaerobes, not tested in this study. Considering triple combinations, ME + CI + FO, ME + CI + MI, and ME + MI + FO demonstrated a significant inhibition of bacterial growth in both planktonic and biofilm states. Clinical case studies have reported positive outcomes with the use of triantibiotic paste (TAP) containing ME + CI + MI at 1 g/mL each for permanent teeth, leading to a reduction in bacterial load and promoting the regeneration of periapical tissues [17–22]. However, it is important to note that the presence of minocycline in the formulation has been linked to tooth discoloration [12,13]. MI causes the chelation of calcium ions, forming calcium-enriched insoluble agglomerates over the dentin surface, as observed by energy-dispersive X-ray spectroscopy analyses [44]. In this study, we utilized a concentration of 5x MBC for each antibiotic, as higher concentrations may pose a risk of toxicity to host cells [45]. Previous studies have incorporated ME + CI + MI combinations at concentrations below 1 g/mL in various vehicles and have demonstrated significant antibiofilm effects [46–49]. The TAP solution eliminated *A. naeslundii* biofilm from root canals and showed a greater efficacy in reducing bacteria-mixed biofilm compared to CH paste and 2% CHX gel, both using CSLM analysis [47,48].

Injectable temperature-sensitive polymers, such as poly-N-vinylcaprolactam (PNVCL), are being explored as potential carriers for various substances, including antibiotics, due to their thermally responsive properties [50–53]. PNVCL has a lower critical solution temperature (LCST) of 33.5 °C, which is below the human body temperature. This characteristic enables it to transition from a liquid to a gel at body temperature, facilitating the controlled release of incorporated substances [31,54]. One study demonstrated the controlled release of ciprofloxacin using the injectable hydrogel HPG-g-PNVCL through spectroscopy, showing promising results for macromolecular drug delivery, supporting the findings of the present study [55]. In another study, it was also observed that the thermosensitive chitosan hydrogel promoted the release of compounds, in addition to increasing their effectiveness with reduced cellular toxicity [26]. Different vehicles have been used for the same purpose as PNVCL hydrogels. Shaik et al. [56] assessed the antimicrobial effectiveness of TAP and calcium hydroxide using chitosan as a carrier against *C. albicans* and *E. faecalis.* They tested four medicaments: TAP + saline, TAP + chitosan, CH + saline, and CH + chitosan. All the treatments demonstrated antifungal and antibacterial properties, with the chitosan-based groups showing superior effects compared to the saline groups [56].

In the present study, MDPC-23 odontoblast-like cells were chosen for the cytotoxicity tests for being a representative odontoblast lineage that express proteins commonly associated with dentin mineralization. Then, they could be suitable in simulating the response of human dental pulp cells during regenerative processes and for evaluating the safety of intracanal medications in human clinical settings. Here, the PNVCL hydrogel diluted 1/8 (25 mg/mL) was not toxic to odontoblast-like cells (MDPC-23), maintaining cell viability above 80%. PNVCL hydrogel was previously evaluated on intestinal (Caco-2) and pulmonary (Calu-3) cells, which showed that, up to 10 mg/mL, it was not detrimental to cell viability [57]. Chondrocytes and mesenchymal stem cells were incorporated into PNVCL hydrogels, and the cytotoxicity analysis revealed high cell viability (>80%) [36]. NHI/3T3 fibroblasts and MDPC-23 odontoblast-like cells were exposed to 48 h and 72 h extracts of serially diluted (from 1/2 to 1/64) PNVCL hydrogels in culture media. The extracts were not toxic to fibroblasts at dilutions below 50% and to MDPC-23 at dilutions below 6.25%, after 24h of exposure [31,52]. Some differences found among the studies are possibly related to the method of hydrogel preparation and the cell lines evaluated. In the present study, PNVCL associated with antibiotics (at 0.625 mg/mL) did not show cell toxicity. This can be explained by the fact that the antibiotics alone at this concentration also promote minimal toxicity. The same explanation can be extended to CHX that was extremely toxic to the MDCP-23 cells above 0.019 mg/mL and, therefore, when associated with the PNVCL hydrogel, also maintained its toxicity. Previous studies have shown that chlorhexidine, even at low concentrations, has shown toxicity on several cell lines, including odontoblast-like cells and fibroblasts, and its prolonged use can lead to adverse effects on the oral mucosa [58–61].

Furthermore, it also important to note that thermoresponsive hydrogels loaded with agents that induce endodontic regeneration have proven to be a promising and biocompatible platform for the sequential release of drugs [51,53]. Although this study did not focus on this application, it provides a foundation for future research in this field. Moving forward, optimizing hydrogel formulations and assessing their in vivo efficacy should be prioritized to improve endodontic treatment outcomes. This study highlights the potential of thermosensitive PNVCL hydrogels as effective antibiotic carriers, offering targeted drug delivery while minimizing systemic exposure. Additionally, the biocompatibility of PNVCL with various cell lines further supports its safety for clinical applications. In addition, the hydrogel (PNVCL with ME + CI + FO) demonstrated antibiofilm effects and could offer a promising alternative to conventional intracanal drugs, particularly for young

permanent teeth. However, this study has some limitations that need to be addressed. The lack of in vivo testing is a significant limitation, as the hydrogel's performance in a real biological environment remains to be tested. In addition, the microbial strains used were limited, focusing mainly on common endodontic pathogens. The inclusion of a wider range of microorganisms would provide a more comprehensive view of the formulation's effectiveness. The wider applicability of this technology could extend beyond immature teeth to mature teeth, offering benefits to patients with persistent infections or resistance to current therapies. However, there are challenges to overcome before extending this formulation for clinical use. Issues relating to cost, long-term stability, and shelf life need to be addressed. In addition, optimizing the hydrogel formulation to ensure consistent quality and efficacy over time will be crucial to its successful integration into clinical practice.

Future investigations should focus on key areas such as gene expression analysis, preclinical trials, and in vivo assessments to further refine therapeutic strategies in endodontics. Understanding the molecular mechanisms underlying the action of thermoresponsive hydrogels containing antibiotics—especially regarding tissue regeneration and drug release dynamics—will offer valuable insights to enhance the precision and success of endodontic treatments. Preclinical and in vivo studies are critical for translating these hydrogel formulations from the laboratory to clinical practice, ensuring their safety, efficacy, and compatibility with human tissues. As these innovative approaches progress, they hold the potential to revolutionize endodontic therapy by facilitating targeted and controlled drug delivery systems, ultimately enhancing patient outcomes and minimizing side effects. A comprehensive research agenda will not only drive the progression of regenerative strategies but also lay the foundation for more personalized and efficient endodontic treatments.

5. Conclusions

Based on the experimental design, it can be concluded that the PNVCL hydrogel effectively delivered the ME + CI + FO combination, demonstrated strong antibiofilm activity, and showed no cytotoxicity towards MDPC-23 odontoblastic cells. These findings emphasize its potential as a promising vehicle for antibiotic delivery in intracanal treatments. Future research should focus on in vivo studies, clinical trials, and broader microbial testing to confirm its clinical efficacy, safety, and potential for broader application in endodontic therapy.

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References

1. Zhang, N.; Kang, Q.; Cheng, Y. Pulpotomy for teeth with irreversible pulpitis in immature permanent teeth: A retrospective case series study. *Sci. Rep.* **2024**, *14*, 6395. [CrossRef]
2. Murray, P.E. Review of guidance for the selection of regenerative endodontics, apexogenesis, apexification, pulpotomy, and other endodontic treatments for immature permanent teeth. *Int. Endod. J.* **2023**, *56*, 188–199. [CrossRef]
3. Gomes, B.P.F.A.; Aveiro, E.; Kishen, A. Irrigants and irrigation activation systems in Endodontics. *Braz. Dent. J.* **2023**, *34*, 1–33. [PubMed]
4. Seron, M.A.; Nunes, G.P.; Ferrisse, T.M.; Strazzi-Sahyon, H.B.; Victorino, F.R.; Dos Santos, P.H.; Gomes-Filho, J.E.; Cintra, L.T.A.; Sivieri-Araujo, G. Postoperative pain after root canal filling with bioceramic sealers: A systematic review and meta-analysis of randomized clinical trials. *Odontology* **2023**, *111*, 793–812. [CrossRef]
5. Pedrinha, V.F.; Barros, M.C.; Portes, J.D.; Slomp, A.M.; Woudstra, W.; Lameira, O.A.; Queiroga, C.L.; Marcucci, M.C.; Shahbazi, M.A.; Sharma, P.K.; et al. Antimicrobial efficacy of alternative root canal disinfection strategies: An evaluation on multiple working models. *Biomed. Pharmacother.* **2025**, *183*, 117833.
6. Wong, J.; Manoil, D.; Näsman, P.; Belibasakis, G.N.; Neelakantan, P. Microbiological Aspects of Root Canal Infections and Disinfection Strategies: An Update Review on the Current Knowledge and Challenges. *Front. Oral Health* **2021**, *2*, 672887.
7. Siqueira, J.F.; Rôças, I.N. Diversity of endodontic microbiota revisited. *J. Dent. Res.* **2009**, *88*, 969–981. [CrossRef] [PubMed]
8. Persoon, I.F.; Buijs, M.J.; Özok, A.R.; Crielaard, W.; Krom, B.P.; Zaura, E.; Brandt, B.W. The mycobiome of root canal infections is correlated to the bacteriome. *Clin. Oral Investig.* **2017**, *21*, 1871–1881. [CrossRef] [PubMed]
9. Yang, S.; Meng, X.; Zhen, Y.; Baima, Q.; Wang, Y.; Jiang, X.; Xu, Z. Srategies and mechanisms targeting Enterococcus faecalis biofilms associated with endodontic infections: A comprehensive review. *Front. Cell. Infect. Microbiol.* **2024**, *14*, 1433313. [CrossRef]
10. Duncan, H.F.; Kirkevang, L.L.; Peters, O.A.; El-Karim, I.; Krastl, G.; Del Fabbro, M.; Chong, B.S.; Galler, K.M.; Segura-Egea, J.J.; Kebschull, M.; et al. Treatment of pulpal and apical disease: The European Society of Endodontology (ESE) S3-level clinical practice guideline. *Int. Endod. J.* **2023**, *56*, 238–295. [CrossRef] [PubMed]
11. Krastl, G.; Weiger, R.; Filippi, A.; Van Waes, H.; Ebeleseder, K.; Ree, M.; Connert, T.; Widbiller, M.; Tjäderhane, L.; Dummer, P.M.H.; et al. Endodontic management of traumatized permanent teeth: A comprehensive review. *Int. Endod. J.* **2021**, *54*, 1221–1245. [CrossRef] [PubMed]
12. da Silva, L.A.B.; Nelson-Filho, P.; da Silva, R.A.B.; Flores, D.S.H.; Heilborn, C.; Johnson, J.D.; Cohenca, N. Revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing in dogs' teeth with apical periodontitis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **2010**, *109*, 779–787. [CrossRef] [PubMed]
13. Acharya, S.; Gurunathan, D.; Singh, B. Triple Antibiotic Paste—Roles and Applications in Pediatric Dentistry. *J. Pharm. Negat. Results* **2023**, *33*, 1375–1378.
14. Lin, J.; Zeng, Q.; Wei, X.; Zhao, W.; Cui, M.; Gu, J.; Lu, J.; Yang, M.; Ling, J. Regenerative Endodontics Versus Apexification in Immature Permanent Teeth with Apical Periodontitis: A Prospective Randomized Controlled Study. *J. Endod.* **2017**, *43*, 1821–1827. [CrossRef]
15. Parhizkar, A.; Nojehdehian, H.; Asgary, S. Triple antibiotic paste: Momentous roles and applications in endodontics: A review. *Restor. Dent. Endod.* **2018**, *43*, e28. [CrossRef]
16. El Ashiry, E.A.; Farsi, N.M.; Abuzeid, S.T.; El Ashiry, M.M.; Bahammam, H.A. Dental Pulp Revascularization of Necrotic Permanent Teeth with Immature Apices. *J. Clin. Pediatr. Dent.* **2016**, *40*, 361–366. [CrossRef]
17. Lillygrace, E.; Kethineni, B.; Puppala, R.; Raichurkar, H.K.; Ambati, S.; Saikiran, K.V. Antimicrobial Efficacy of Triple Antibiotic Paste and Propolis as an Intracanal Medicament in Young Permanent Teeth: An In Vivo Study. *Int. J. Clin. Pediatr. Dent.* **2021**, *14*, 243–248. [CrossRef]
18. Trope, M. Treatment of the immature tooth with a non-vital pulp and apical periodontitis. *Dent. Clin. N. Am.* **2010**, *54*, 313–324. [CrossRef] [PubMed]
19. Hoshino, E.; Kurihara-Ando, N.; Sato, I.; Uematsu, H.; Sato, M.; Kota, K.; Iwaku, M. In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. *Int. Endod. J.* **1996**, *29*, 125–130. [CrossRef] [PubMed]
20. Machado, J.C. Combined Effect of Antibiotics and Simvastatin on Endodontic Microorganisms and the Expression of Odontoblast Markers. Master´s Thesis, Universidade Estadual Paulista, São Paulo, Brazil, 2016. Available online: http://acervodigital.unesp.br/handle/11449/138844 (accessed on 2 February 2025).
21. Mandras, N.; Roana, J.; Allizond, V.; Pasqualini, D.; Crosasso, P.; Burlando, M.; Banche, G.; Denisova, T.; Berutti, E.; Cuffini, A.M. Antibacterial efficacy and drug-induced tooth uration of antibiotic combinations for endodontic regenerative procedures. *Int. J. Immunopathol. Pharmacol.* **2013**, *26*, 557–563. [CrossRef]

22. Duque, C.; Souza, A.C.A.; Aida, K.L.; Pereira, J.A.; Caiaffa, K.S.; Santos, V.R.D.; Cosme-Silva, L.; Prakki, A. Synergistic antimicrobial potential of EGCG and fosfomycin against biofilms associated with endodontic infections. *J. Appl. Oral Sci.* **2023**, *31*, e20220282. [CrossRef] [PubMed]
23. Althumairy, R.I.; Teixeira, F.B.; Diogenes, A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. *J. Endod.* **2014**, *40*, 521–525. [CrossRef]
24. Malu, K.; Khubchandani, M. Triple Antibiotic Paste: A Suitable Medicament for Intracanal Disinfection. *Cureus* **2022**, *14*, e29186. [CrossRef] [PubMed]
25. Pereira, A.C.C.; Aguiar, A.P.S.; Barbosa, V.L.; Régis, J.R.; Miyazima, E.M.; Araujo, L.M.P.; Dantas, L.O.; Mayer, M.P.A.; Andrade, F.B.; Karygianni, L.; et al. Enhancing Antibiotic Efficacy in Regenerative Endodontics by Improving Biofilm Susceptibility. *J. Endod.* **2024**, *50*, 962–965. [CrossRef]
26. Li, S.; Dong, S.; Xu, W.; Tu, S.; Yan, L.; Zhao, C.; Ding, J.; Chen, X. Antibacterial hydrogels. *Adv. Sci.* **2018**, *5*, 1700527. [CrossRef] [PubMed]
27. Atila, D.; Kumaravel, V. Advances in antimicrobial hydrogels for dental tissue engineering: Regenerative strategies for endodontics and periodontics. *Biomater. Sci.* **2023**, *11*, 6711–6747. [CrossRef]
28. Leveque, M.; Bekhouche, M.; Farges, J.C.; Aussel, A.; Sy, K.; Richert, R.; Ducret, M. Bioactive Endodontic Hydrogels: From Parameters to Personalized Medicine. *Int. J. Mol. Sci.* **2023**, *24*, 14056. [CrossRef]
29. Gonzalez-Urias, A.; Licea-Claverie, A.; Sañudo-Barajas, J.A.; González-Ayón, M.A. NVCL-Based Hydrogels and Composites for Biomedical Applications: Progress in the Last Ten Years. *Int. J. Mol. Sci.* **2022**, *23*, 4722. [CrossRef] [PubMed]
30. Halligan, E.; Zhuo, S.; Colbert, D.M.; Alsaadi, M.; Tie, B.S.H.; Bezerra, G.S.N.; Keane, G.; Geever, L.M. Modulation of the Lower Critical Solution Temperature of Thermoresponsive Poly(N-vinylcaprolactam) Utilizing Hydrophilic and Hydrophobic Monomers. *Polymers* **2023**, *15*, 1595. [CrossRef]
31. Braga, G.P.A.; Caiaffa, K.S.; Pereira, J.A.; Santos, V.R.D.; Souza, A.C.A.; Ribeiro, L.D.S.; Camargo, E.R.; Prakki, A.; Duque, C. Microbiological Properties and Cytotoxicity of PNVCL Hydrogels Containing Flavonoids as Intracanal Medication for Endodontic Therapy. *J. Funct. Biomater.* **2022**, *13*, 305. [CrossRef]
32. *CLSI M07*; Methos for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; 11th ed. Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2012.
33. *CLSI M27*; Reference Methos For Broth Dilution Antifungal Susceptibility Testing of Yeasts; 4th ed. Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2017.
34. Jacob, V.P.; Paião, L.I.; da Silva, A.C.G.; Magario, M.K.W.; Kaneko, T.Y.; Martins, C.M.; Monteiro, D.R.; Mori, G.G. Antimicrobial action of NeoMTA Plus on mono- and dual-species biofilms of *Enterococcus faecalis* and *Candida albicans*: An in vitro study. *Arch. Oral Biol.* **2020**, *120*, 104925. [CrossRef]
35. Li, W.; Liu, H.; Xu, Q. Extracellular dextran and DNA affect the formation of Enterococcus faecalis biofilms and their susceptibility to 2% chlorhexidine. *J. Endod.* **2012**, *38*, 894–898. [CrossRef] [PubMed]
36. Sala, R.L.; Kwon, M.Y.; Kim, M.; Gullbrand, S.E.; Henning, E.A.; Mauck, R.L.; Camargo, E.R.; Burdick, J.A. Thermosensitive Poly(N-vinylcaprolactam) Injectable hydrogels for cartilage tissue engineering. *Tissue Eng. Part A* **2017**, *23*, 935–945. [CrossRef] [PubMed]
37. Caiaffa, K.S.; Basso, F.G.; Santos-Filho, N.A.; de Souza-Costa, C.A.; Sakai, V.T.; Cilli, E.M.; Duque, C. Effect of analogues of cationic peptides on dentin mineralization markers in odontoblast-like cells. *Arch. Oral Biol.* **2019**, *103*, 19–25. [CrossRef] [PubMed]
38. Massunari, L.; Rabelo, R.L.; Leite, M.L.; Soares, D.G.; Anovazzi, G.; Costa, C.A.S.; Duque, C. Dose- and time-dependent effects of taxifolin on viability and mineralization markers of osteoblast-like cells. *Braz. Oral Res.* **2021**, *35*, e140. [CrossRef] [PubMed]
39. Smith, A.J.; Hall, V.; Thakker, B.; Gemmell, C.G. Antimicrobial susceptibility testing of *Actinomyces* species with 12 antimicrobial agents. *J. Antimicrob. Chemother.* **2005**, *56*, 407–409. [CrossRef] [PubMed]
40. LeCorn, D.W.; Vertucci, F.J.; Rojas, M.F.; Progulske-Fox, A.; Bélanger, M. In vitro activity of amoxicillin, clindamycin, doxycycline, metronidazole, and moxifloxacin against oral Actinomyces. *J. Endod.* **2007**, *33*, 557–560. [CrossRef] [PubMed]
41. Keepers, T.R.; Gomez, M.; Celeri, C.; Krause, K.M.; Biek, D.; Critchley, I. Fosfomycin and comparator activity against select Enterobacteriaceae, *Pseudomonas*, and *Enterococcus* urinary tract infection isolates from the United States in 2012. *Infect. Dis. Ther.* **2017**, *6*, 233–243. [CrossRef] [PubMed]
42. Kaushik, S.N.; Scoffield, J.; Andukuri, A.; Alexander, G.C.; Walker, T.; Kim, S.; Choi, S.C.; Brott, B.C.; Eleazer, P.D.; Lee, J.-Y.; et al. Evaluation of ciprofloxacin and metronidazole encapsulated biomimetic nanomatrix gel on Enterococcus faecalis and Treponema denticola. *Biomater. Res.* **2015**, *19*, 9. [CrossRef]
43. Tang, H.J.; Chen, C.C.; Zhang, C.C.; Su, B.A.; Li, C.M.; Weng, T.C.; Chiang, S.-R.; Ko, W.-C.; Chuang, Y.-C. In vitro efficacy of fosfomycin-based combinations against clinical vancomycin-resistant Enterococcus isolates. *Diagn. Microbiol. Infect. Dis.* **2013**, *77*, 254–257. [CrossRef]
44. Porter, M.L.; Münchow, E.A.; Albuquerque, M.T.; Spolnik, K.J.; Hara, A.T.; Bottino, M.C. Effects of novel 3-dimensional antibiotic-containing electrospun scaffolds on dentin discoloration. *J. Endod.* **2016**, *42*, 106–112.

45. Muteeb, G.; Rehman, M.T.; Shahwan, M.; Aatif, M. Origin of Antibiotics and Antibiotic Resistance, and Their Impacts on Drug Development: A Narrative Review. *Pharmaceuticals* **2023**, *16*, 1615. [CrossRef]
46. Valan, A.S.; Kolli, S.; Eswaramoorthy, R.; Krithikadatta, J.; Malli Sureshbabu, N. Comparison of Antibacterial Efficacy of Triple Antibiotic-Loaded Hydrogel Versus Modified Triple Antibiotic-Loaded Hydrogel as Intracanal Medicament Against Enterococcus faecalis: An In vitro Study. *Eur. Endod. J.* **2024**, *9*, 154–160. [PubMed]
47. Ordinola-Zapata, R.; Bramante, C.M.; Minotti, P.G.; Cavenago, B.C.; Garcia, R.B.; Bernardineli, N.; Jaramillo, D.E.; Duarte, M.A.H. Antimicrobial activity of triantibiotic paste, 2% chlorhexidine gel, and calcium hydroxide on an intraoral-infected dentin biofilm model. *J. Endod.* **2013**, *39*, 115–118. [CrossRef] [PubMed]
48. Albuquerque, M.T.; Ryan, S.J.; Münchow, E.A.; Kamocka, M.M.; Gregory, R.L.; Valera, M.C.; Bottino, M.C. Antimicrobial effects of novel triple antibiotic paste-mimic scaffolds on Actinomyces naeslundii biofilm. *J. Endod.* **2015**, *41*, 1337–1343.
49. Smittiset, B.; Banomyong, D.; Ruangsawasdi, N.; Kaewprag, J. In vitro bactericidal efficacy of a new triple antibiotic paste formulation against Enterococcus faecalis biofilm. *Aust. Endod. J.* **2023**, *49*, 9–17. [CrossRef]
50. Tucker, L.J.; Grant, C.S.; Gautreaux, M.A.; Amarasekara, D.L.; Fitzkee, N.C.; Janorkar, A.V.; Varadarajan, A.; Kundu, S.; Priddy, L.B. Physicochemical and Antimicrobial Properties of Thermosensitive Chitosan Hydrogel Loaded with Fosfomycin. *Mar. Drugs* **2021**, *19*, 144. [CrossRef]
51. Maddeppungeng, N.M.; Syahirah, N.A.; Hidayati, N.; Rahman, F.U.A.; Mansjur, K.Q.; Rieuwpassa, I.E.; Setiawati, D.; Fadhlullah, M.; Aziz, A.Y.R.; Salsabila, A.; et al. Specific Delivery of Metronidazole Using Microparticles and Thermosensitive In Situ Hydrogel for Intrapocket Administration as an Alternative in Periodontitis Treatment. *J. Biomater. Sci. Polym. Ed.* **2024**, *35*, 1726–1749. [CrossRef] [PubMed]
52. Braga, G.P.A.; Caiaffa, K.S.; Rabelo, R.L.; Santos, V.R.D.; Souza, A.C.A.; Ribeiro, L.D.S.; Camargo, E.R.; Prakki, A.; Duque, C. Cytotoxicity and Biomineralization Potential of Flavonoids Incorporated into PNVCL Hydrogels. *J. Funct. Biomater.* **2023**, *14*, 139. [CrossRef] [PubMed]
53. Sun, M.; Zhu, C.; Long, J.; Lu, C.; Pan, X.; Wu, C. PLGA Microsphere-Based Composite Hydrogel for Dual Delivery of Ciprofloxacin and Ginsenoside Rh2 to Treat *Staphylococcus aureus*-Induced Skin Infections. *Drug Deliv.* **2020**, *27*, 632–641. [CrossRef] [PubMed]
54. Shamszadeh, S.; Asgary, S.; Akrami, M.; Mashhadiabbas, F.; Akbarzadeh Baghban, A.; Shams, F. Development of a Thermoresponsive Core-Shell Hydrogel for Sequential Delivery of Antibiotics and Growth Factors in Regenerative Endodontics. *Front. Biosci. (Elite Ed.)* **2024**, *16*, 32.
55. Parameswaran-Thankam, A.; Parnell, C.M.; Watanabe, F.; RanguMagar, A.B.; Chhetri, B.P.; Szwedo, P.K.; Biris, A.S.; Ghosh, A. Guar-based injectable thermoresponsive hydrogel as a scaffold for bone cell growth and controlled drug delivery. *ACS Omega* **2018**, *3*, 15158–15167. [PubMed]
56. Shaik, J.; Garlapati, R.; Nagesh, B.; Sujana, V.; Jayaprakash, T.; Naidu, S. Comparative evaluation of antimicrobial efficacy of triple antibiotic paste and calcium hydroxide using chitosan as a carrier against *Candida albicans* and *Enterococcus faecalis*: An in vitro study. *J. Conserv. Dent.* **2014**, *17*, 335–339. [PubMed]
57. Vihola, H.; Laukkanen, A.; Valtola, L.; Tenhu, H.; Hirvonen, J. Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam). *Biomaterials* **2005**, *26*, 3055–3064. [CrossRef]
58. Lessa, F.C.; Aranha, A.M.; Nogueira, I.; Giro, E.M.; Hebling, J.; Costa, C.A. Toxicity of chlorhexidine on odontoblast-like cells. *J. Appl. Oral Sci.* **2010**, *18*, 50–58. [PubMed]
59. Kreling, P.F.; Aida, K.L.; Massunari, L.; Caiaffa, K.S.; Percinoto, C.; Bedran, T.B.; Spolidorio, D.M.P.; Abuna, G.F.; Cilli, E.M.; Duque, C. Cytotoxicity and the effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions. *Biofouling* **2016**, *32*, 995–1006. [CrossRef]
60. Caiaffa, K.S.; Massunari, L.; Danelon, M.; Abuna, G.F.; Bedran, T.B.L.; Santos-Filho, N.A.; Spolidorio, D.M.P.; Vizoto, N.L.; Cilli, E.M.; Duque, C. KR-12-a5 is a non-cytotoxic agent with potent antimicrobial effects against oral pathogens. *Biofouling* **2017**, *33*, 807–818.
61. Dinu, S.; Matichescu, A.; Buzatu, R.; Marcovici, I.; Geamantan-Sirbu, A.; Semenescu, A.D.; Bratu, R.C.; Bratu, D.C. Insights into the cytotoxicity and irritant potential of chlorhexidine digluconate: An in vitro and in ovo safety screening. *Dent. J.* **2024**, *12*, 221. [CrossRef] [PubMed]

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