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Research Article

Leveraging Bacteriophages to Mitigate *Enterococcus faecalis* Re-infection in Endodontic Procedures: An *In-vitro* and *Ex-vivo* Study

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Abstract

Introduction: This study was designed to assess the efficacy of bacteriophages against *Enterococcus faecalis (E. faecalis)* biofilms formed on root canal surfaces.

Methodology: A biofilm of *E. faecalis* was developed in 96-well polystyrene plates using BHI broth and incubated for seven days. Wells were divided into five groups: control, normal saline, PBS, 1% sodium hypochlorite and bacteriophage, with each exposed respective compositions overnight. After treatment, wells were washed with PBS, stained with 0.5% crystal violet and absorbance was measured at 570 nm. In the *ex-vivo* phase, extracted human teeth underwent root canal preparation and *E. faecalis* biofilm induction. After seven days, teeth were treated as per their group, rinsed with PBS and filled with saline. Samples were collected using paper points, vortexed, plated on MHA and CFU/mL were calculated for statistical analysis.

Results: *In-vitro*, bacteriophage treatment significantly reduced biofilm mass (OD: 0.17 ± 0.04) vs. control (0.95 ± 0.16), similar to 1% NaOCl (0.10 ± 0.04). In the *ex-vivo* model, CFU counts were highest in the control (135,468.14 ± 47,922.08), markedly reduced after single bacteriophage application (1,528.57 ± 1,932.80) and completely eradicated after the second one.

Conclusion: Bacteriophages exhibit a destructive effect against *E. faecalis* in biofilm form within root canals and may be considered a part of alternative antimicrobial strategies to combat biofilm-associated endodontic infections.

Keywords: *Enterococcus faecalis*; Biofilm; Endodontic Infections; Bacteriophage; Bacteriophage -Therapy; Intra-Canal Disinfectant

Introduction

The involvement of microbes in dental diseases is attributable to the existence of over 700 bacterial species in an oral cavity [1]. Recurrent or persistent endodontic infections pose a significant challenge for both clinicians and patients, largely due to the complex anatomy of the root canal system and the resilient biofilm-forming capabilities of microorganisms. This persistent infection eventually leads to toothache and discomfort, which hampers the quality of life. Hence, managing the causative microbes thoroughly and efficiently is of utmost importance.

One of the key factors enabling microbial persistence in root canals is the complex anatomy of the root canal system, including apical deltas and narrow isthmuses. These anatomical irregularities hinder complete mechanical debridement, necessitating the use of chemical disinfectants in conjunction with instrumentation [2]. In response, dentistry has seen a surge in the use of various antiseptics, disinfectants and irrigation techniques. Commonly used agents include sodium hypochlorite (NaOCl), citric acid, EDTA, maleic acid, etidronic acid, chlorine dioxide, silver diamine fluoride, chlorhexidine, Mixture of Tetracycline isomer, Acid and Detergent (MTAD), herbal extracts (e.g., green tea, Morinda citrifolia) and antibiotic solutions (double and triple combinations) [3]. Additionally, certain NSAIDs like diclofenac (1.25%-5%) have demonstrated antimicrobial activity, particularly against biofilms [4]. These agents are often used in combination for synergistic action some targeting organic debris while others focus on inorganic matter. However, limitations exist. While NaOCl is considered the gold standard, higher concentrations (e.g., 5.25%) can weaken dentin due to their erosive action and increase the risk of hypochlorite accidents [5,6]. Furthermore, antibiotics may face the risk of resistance and many disinfectants can negatively impact dentin integrity.

Moreover, despite the wide array of available disinfectants, Apical Periodontitis (AP) is prevalent in up to 41.3% of Endodontically Treated Teeth (ETT) globally [7]. Among the microbial culprits, *Enterococcus faecalis (E. faecalis*)-a gram-positive gut commensal is particularly prevalent, in 90% of such cases [9]. Known for its ability to withstand harsh environmental conditions, *E. faecalis* contributes to periapical tissue damage primarily through host-mediated responses [10]. It possesses surface adhesion properties and a strong ability to form biofilm, enhancing its resistance to treatment [11]. Biofilms, which are dense and multilayered structures, often limit the efficacy of conventional disinfectants and antibiotics. They represent a particularly resilient form of bacterial colonization and are commonly implicated in root canal infections.

In contrast, bacteriophages-natural predators of bacteria can disrupt biofilms using several enzymes, enhancing the penetration and effectiveness of therapeutic agents; when combined with antibiotics, they mitigate antibiotic resistance and improve bacterial eradication [12,13]. Given these advantages, this study aims to evaluate the efficacy of bacteriophage therapy in eradicating *E. faecalis* biofilms formed on dentinal surfaces.

Methodology

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines. This study was carried out in the Viral Research and Diagnostic Laboratory (VRDL), Department of Microbiology, of our institute. The institutional ethical committee approved the study with the reference number Dean/2022/EC/3330.

Test Organism

A clinical strain of *E. faecalis* was obtained from the Department of Microbiology, IMS, BHU. This strain was confirmed as *E. faecalis* by using the standard biochemical and molecular methods. The bacterial strain was inoculated in the brain heart infusion broth (BHI, Himedia, Mumbai, India) and incubated at 37°C overnight. The experimental suspensions were prepared as per the following protocol.

Isolation and Purification of Bacteriophages Against the Experimental Bacterial Strain

Bacteriophages were isolated from various water sources (rivers, ponds and sewers) as described by Lee, et al. [14]. Briefly, 10 mL of water was mixed with 40 mL of SM buffer (100 mM NaCl, 10 mM $MgSO_4·7H_2O$, 50 mM Tris-HCl, pH 7.5) and 1% chloroform, then centrifuged (10,000 × g, 4°C, 10 min). The supernatant was filtered (0.22 μm), mixed with 2x BHI broth and *E. faecalis* at the concentration of 1x 108 cfu/mL in log phase and incubated overnight at 37°C. After re-centrifugation and filtration, the phage suspension was serially diluted and spotted on soft agar seeded with *E. faecalis*. Clear plaques were picked, resuspended in SM buffer and purified through repeated soft agar overlays [15].

To obtain purified, concentrated phage, the lysate was dialyzed using 0.02 μm membrane with 20% PEG 6000 in 2.5 M NaCl hypertonic solution outside, followed by PBS washing to eliminate bacterial contaminants [16]. Phage activity was confirmed by spotting 10 μL of 10^9 PFU/mL onto an *E. faecalis* lawn, producing clear lytic zones. Phage morphology was further visualized using Transmission Electron Microscopy (TEM) (Fig. 1).

Experiment Set-Up: Biofilm Assay on Microtitre Plate

A bacterial suspension of *E. faecalis* (1 McFarland unit) was prepared from a fresh clinical isolate and diluted tenfold in nutrient broth [17]. Biofilm assays were conducted using a sterile 96-well polystyrene microtiter plate to evaluate bacteriophage efficacy against *E. faecalis* biofilms, compared with 1% sodium hypochlorite (NaOCl), selected for its balance of antimicrobial efficacy and reduced cytotoxicity [18]. As shown in Fig. 2, peripheral wells were filled with sterile PBS as sterility controls, while inner wells received 150μL of the bacterial suspension and were incubated at 37°C for 7 days, with replenishment with nutrient broth every 72 hours.

After incubation, wells were gently aspirated and washed with PBS to remove planktonic cells. The wells were then divided into five groups: control, normal saline, PBS, 1% NaOCl and bacteriophage (10^9 PFU/mL). Each group received 180 μL of the respective solution and was incubated overnight (Fig. 2). The next day, wells were washed with PBS, stained with 0.5% crystal violet for 30 minutes, rinsed with distilled water and air-dried overnight. Biofilm quantification was done by adding 200μL of 30% acetic acid to each well (Fig. 3) and absorbance was measured at 570 nm using a spectrophotometer (Thermo Fisher Scientific, MULTISKAN FC). Unstained wells treated with acetic acid served as blanks. The entire experiment was repeated to ensure the reproducibility.

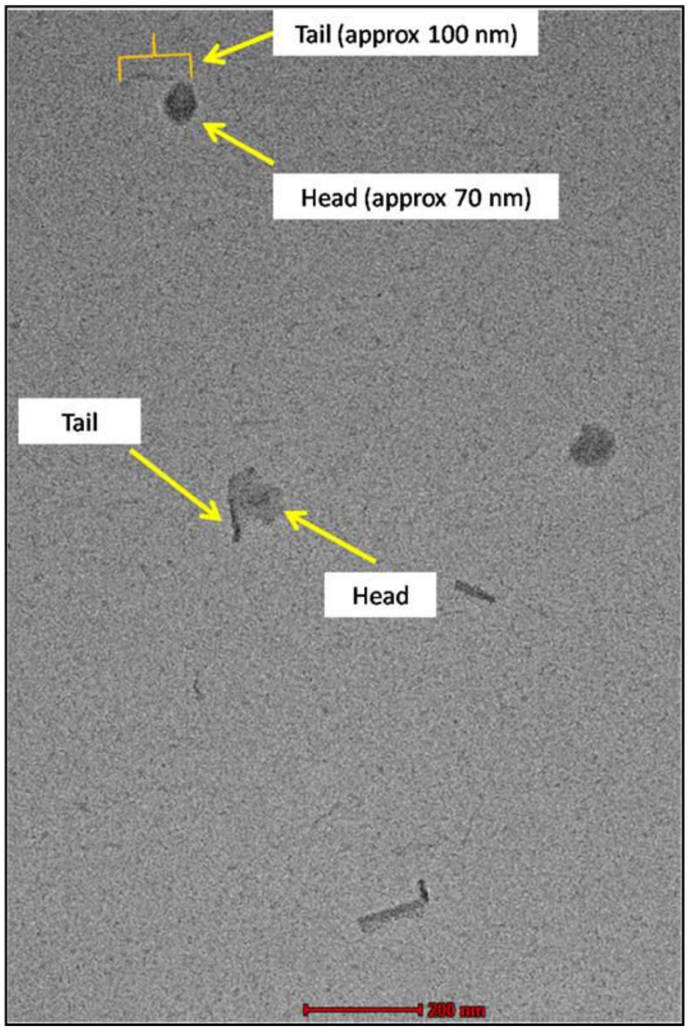

Figure 1: Transmission Electron Microscopy (TEM) image of bacteriophage against *E. faecalis*, showcasing the distinct morphology of two phages. The hexagonal head measures approximately 70 nm in diameter, with a tail length of about 100 nm.

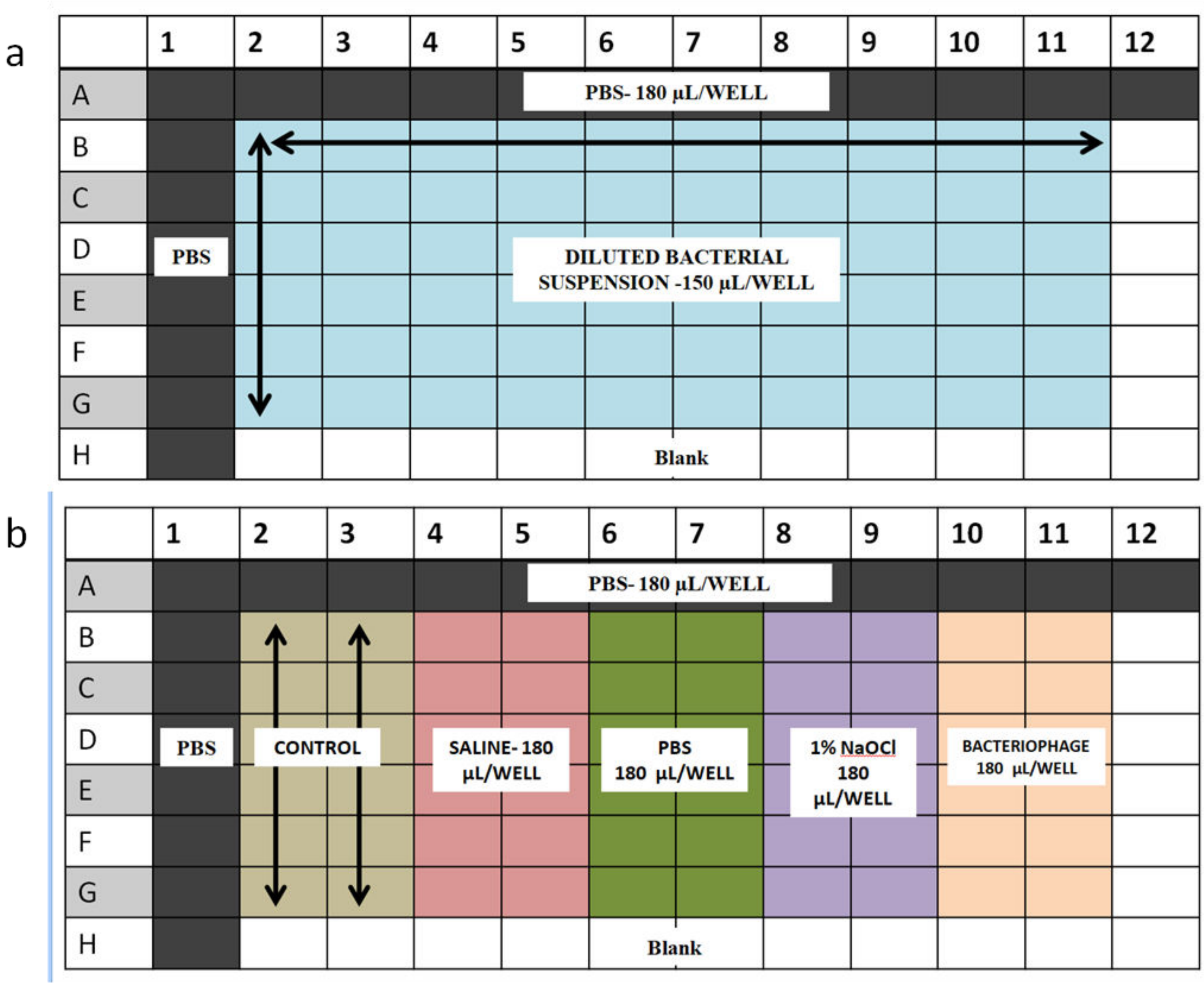

Figure 2: (a): Pictorial representation of well-allotment for inducing biofilm formation in a polystyrene microtiter plate. (b): Pictorial representation of well-allotment to different reagents after biofilm formation in polystyerene microtitre plate.

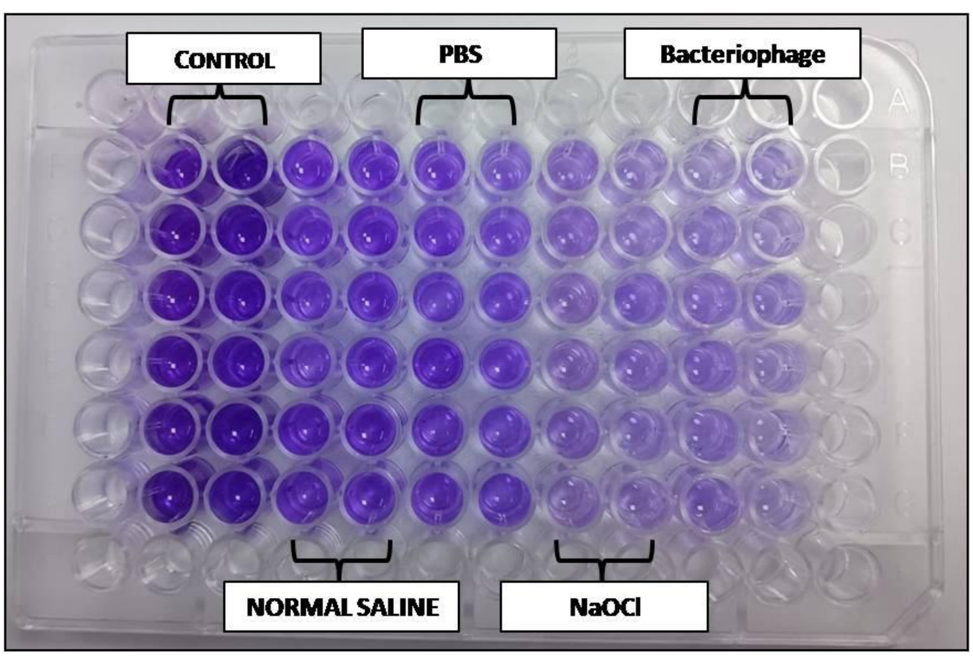

Figure 3: Polystyerene microtitre plate (96 wells) showing antibacterial activity of different agents.

Tooth Model Biofilm Assay

Tooth Selection and Preparation

Extracted human maxillary premolars with fully developed roots and single, straight canals were collected from dental clinics in Varanasi and stored in 3% hydrogen peroxide. Teeth with intact cementum and no residual bone, calculus or soft tissue were selected and cleaned using curettes, then stored in sterile saline.

Canal Preparation

Access cavities were prepared and canals were instrumented up to size 50 K-file (Dentsply Maillefer). During preparation, canals were irrigated with 3 mL of 1% NaOCl at each file change. After a final rinse with NaOCl, canals were flushed with sterile saline to remove residues and dried using size 20 paper points. Sterility was confirmed by culturing these points before proceeding.

Biofilm Formation

A suspension of *E. faecalis* (3×10^7 CFU/mL) was inoculated into the prepared canals using a 27-gauge needle. Apical ends were sealed with parafilm and teeth were mounted on a sterile stand and incubated at 37°C for 7 days. The bacterial suspension was replenished daily.

Treatment Protocol

After 7 days, canals were aspirated and dried. Teeth were randomly divided into five groups:

- Group A: Control
- Group B: Normal saline (0.9%)
- Group C: PBS
- Group D: 1% NaOCl
- Group E: Bacteriophage (10^9 PFU/mL)

Experimental agents were introduced into the canals using sterile 27-gauge needles and incubated overnight at 37°C.

Sample Collection and Analysis

Reagents were aspirated and canals were rinsed with PBS and saline. A size 10 K-file was used to agitate the canal walls, followed by insertion of a sterile size 20 paper point for 1 minute. Paper points were transferred to microcentrifuge tubes with 1 mL saline, vortexed for 30 seconds and plated on MHA plates. Additionally, 100 μL of the solution was used for quantification after overnight incubation at 37°C. In Group E, teeth that exhibited bacterial growth after initial treatment were retreated with bacteriophage suspension and incubated overnight at 37°C. The next day, canals were rinsed with sterile PBS and samples were collected as described previously. These were plated on MHA and incubated overnight to assess the presence of bacterial colonies (Fig. 4).

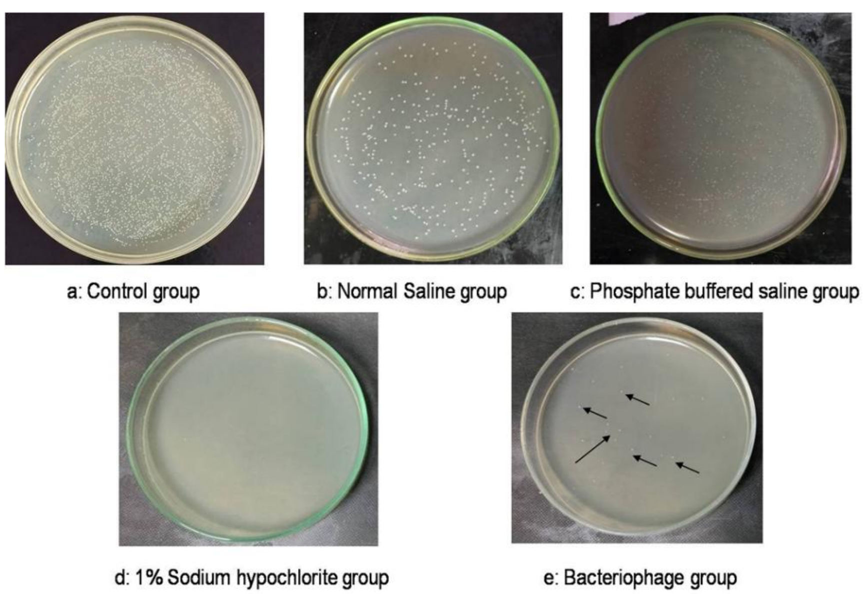

Figure 4: Representative images of all the 5 experimental groups. Petri-plates depicting growth of *E. faecalis* after 1st round of application of different agents in the root canals infected with *E. faecalis* bacteria.

Statistical Analysis

The data obtained was entered into a Microsoft Excel Worksheet and analyzed using Statistical Package for Social Sciences (SPSS, IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.). The normal distribution of data was assessed with Shapiro-Wilk's test and appropriate parametric tests were employed. Comparison of mean OD values amongst 5 study groups by using One-way ANOVA followed by Post Hoc Pairwise Multiple Comparisons with Bonferroni correction. Non-parametric statistics were applied on CFU count amongst 4 study groups using the Kruskal-Wallis test followed by Post Hoc Pairwise Multiple Comparisons with Bonferroni correction. A $p<0.05$ was considered significant for all statistical inferences.

Results

Bacteriophages targeting a clinical strain of *E. faecalis* were isolated and confirmed using Transmission Electron Microscopy (TEM), which revealed a distinct morphology characterized by a hexagonal head approximately 70 nm in diameter and a tail around 100 nm in length (Fig. 1).

In-vitro Polystyrene 96-Well Microtiter Plates Results

Differences in crystal violet staining intensity among the control, bacteriophage and NaOCl groups were visibly apparent to the naked eye (Fig. 3). The bacterial density in each well was quantified by measuring the Optical Density (OD), with higher OD values indicating higher bacterial counts. The control group exhibited the highest OD values (0.95 ± 0.16), while the NaOCl group showed the lowest (0.10 ± 0.04) (Table 1). The OD value for the bacteriophage group (0.17 ± 0.04) was slightly higher than that of the NaOCl group; however, the difference was not statistically significant ($p = 0.29$) (Table 2).

No significant differences were observed among the OD values of the normal saline (0.86 ± 0.12), PBS (0.89 ± 0.16) and control (0.95 ± 0.16) groups, with p-values of 0.06 and 0.4, respectively (Table 1,2) indicating similar bacterial load in these wells. Multiple comparison analysis revealed that both the NaOCl and bacteriophage groups demonstrated significantly lower OD values compared to the control group, while all other groups showed no significant differences when compared with each other (Table 3).

Ex-vivo Study Results

After the first round of exposure of *E. faecalis* biofilm (formed within the root canals) to the respective agents, the following observations were made:

Bacterial growth was evident on all plates from Groups A, B and C (Fig. 4,5). However, a visibly lower density of colonies was observed in Groups B and C compared to Group A. Group D demonstrated complete sterility, with all plates showing no bacterial growth and CFU/mL values recorded as zero. In Group E, 57% of the plates showed no bacterial growth, while 43% were non-sterile and exhibited a few bacterial colonies. Despite this, the CFU/mL count in the 43% of non-sterile Group E samples (1528.57 ± 1932.8) was significantly lower than that of the control group, Group A (135,468.14 ± 47,922.08), with a p-value of <0.001 (Table 4).

Following a second round of exposure applied to the root canals corresponding to the 43% of non-sterile Group E samples, no bacterial colonies were observed on the plates, indicating complete sterilization.

Groups	Mean±SD	f	p-value
Control (BHI plus bacteria)	0.95±0.16	298.46	<0.001
Normal Saline	0.86±0.12		
Phosphate buffered saline	0.89±0.16		
1 % Sodium Hypochlorite	0.1±0.04		
Bacteriophage 10^9 pfu/ml	0.17±0.04		

Table 1: Mean values of optical densities of experimental groups.

Groups	Comparison with	Mean Difference	*p*	95% Confidence Interval
Control (BHI plus bacteria)	Normal Saline	0.09	0.06	-0.01 to 0.18
	Phosphate buffered saline	0.06	0.4	-0.03 to 0.15
	1 % Hypochlorite	0.84	**<0.001**	0.75 to 0.94
	Bacteriophage 10^9 pfu/ml	0.78	**<0.001**	0.68 to 0.87
Normal Saline	Phosphate buffered saline	-0.03	0.87	-0.13 to 0.06
	1 % hypo	0.75	**<0.001**	0.66 to 0.85
	Bacteriophage 10^9 pfu/ml	0.69	**<0.001**	0.59 to 0.78
Phosphate buffered saline	1% Sodium Hypochlorite	0.78	**<0.001**	0.69to 0.88
	Bacteriophage 10^9 pfu/ml	0.72	**<0.001**	0.62 to 0.81
1% Sodium Hypochlorite	Bacteriophage 10^9 pfu/ml	-0.06	0.29	-0.16 to 0.03

Table 2: Post-hoc test with Bonferroni Correction for multiple comparisons of OD values of different experimental groups of Table 1.

Group	Mean±SD CFU/mL	Kruskal-Wallis Test	p-value
Control (BHI plus bacteria)	135468.14±47922.08	23.25	**<0.001**
Normal Saline	21842.86±7405.37		
Phosphate buffered saline	14733.33±5662.39		
Bacteriophage 10^9 pfu/ml	1528.57±1932.8		

Table 3: Mean values of colony forming units obtained after 1st round of treatment with different agents of respective experimental groups.

Group	Comparison	Test statistics	p-value
Control (BHI plus bacteria)	Normal Saline	1.89	0.35
	Phosphate buffered saline	2.79	0.03
	Bacteriophage 10^9 pfu/ml	4.72	<0.001
Normal Saline	Phosphate buffered saline	0.98	0.9
	Bacteriophage 10^9 pfu/ml	2.83	0.02
PBS	Bacteriophage 10^9 pfu/ml	1.74	0.49

Table 4: Post hoc test with Bonferroni Correction for multiple comparisons for mean values of CFU/mL of Table 3.

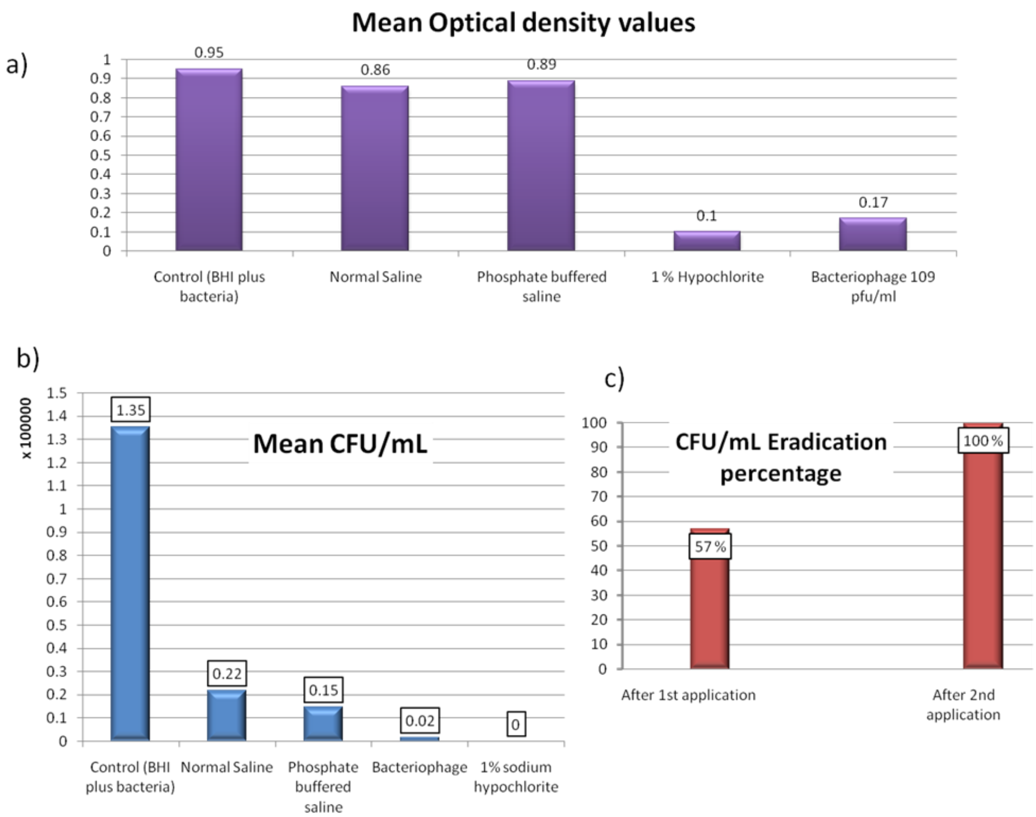

Figure 5: (a): The mean optical density values for all experimental groups on the polystyrene plate, recorded at 570 nm. (b): The mean colony-forming units (CFU/mL) observed on culture plates after the first round of treatment with the respective reagents in each experimental group. (c): The percentage of extracted teeth samples that showed complete eradication of bacteria from the root canals after subsequent rounds of bacteriophage treatment.

Discussion

In light of the advantages that bacteriophages offer over currently available root canal disinfectants and antiseptics, this study was designed to evaluate the efficacy of specific bacteriophages in eradicating *Enterococcus faecalis* in both its planktonic and biofilm forms.

Notably, in the *ex-vivo* model, two subsequent applications of the phage suspension were sufficient to completely eradicate the bacterial biofilm-an outcome typically achieved with a single application of NaOCl. Although NaOCl is widely regarded for its broad-spectrum antimicrobial efficacy, its known cytotoxicity to dentinal and periradicular tissues limits its biocompatibility [18]. In contrast, bacteriophages demonstrated effective biofilm eradication without exhibiting such undesirable tissue-irritating effects.

One of the key advantages of bacteriophages lies in their ability to penetrate thick biofilms, a task with which NaOCl may achieve but with erosion of soft tissues. This is facilitated by phage-encoded enzymes, including polysaccharide depolymerases that degrade the extracellular matrix and peptidoglycan hydrolases that disrupt the bacterial cell wall from within [12]. Supporting our findings, prior studies in non-endodontic contexts have also demonstrated the robust anti-biofilm activity of bacteriophages across various bacterial species [17,19]. Furthermore, Pei, et al., reported that phages can induce quorum quenching through lactonase production, thereby inhibiting biofilm formation at its early stages [19].

Beyond their direct lytic action, bacteriophages offer several immunological advantages. They enhance bacterial clearance by promoting opsonization, rendering pathogens more susceptible to phagocytosis by immune cells such as macrophages. Remarkably, phages may continue their lytic activity intracellularly during phagocytosis. Some studies have also highlighted their potential immunomodulatory effects. While impure phage preparations may provoke mild cytokine responses, purified bacteriophages generally elicit minimal or no inflammatory reactions. Both human and animal studies have shown reductions in inflammatory markers following phage therapy, likely due to effective bacterial eradication and its anti-inflammatory effect. In addition, certain phages appear to influence immune signaling pathways, although the exact mechanisms are not yet fully understood [20].

Bacteriophage therapy offers synergy with local antibiotics, enhancing bactericidal efficacy in chronic periapical lesions [21]. Unlike conventional disinfectants, phages self-replicate in the presence of their host and can eliminate dormant persister cells upon reactivation [22].

A significant advantage of bacteriophage therapy is its high specificity, which allows it to target pathogenic bacteria without disturbing the beneficial microbiota of the oral cavity. While a single application may be effective, our findings suggest that multiple doses could further optimize root canal disinfection. Additionally, as bacteriophages are natural components of the human microbiome, repeated applications have not been shown to elicit immune responses for up to three weeks post-administration [20,23,24]. Thus, bacteriophages can be regarded as a potent tool in combating the continual rise of antimicrobial resistance [25].

However, it is important to recognize the limitations of bacteriophage therapy. Phages do not assist in removing pulpal remnants or inorganic debris, which are critical components of root canal disinfection. These limitations can be addressed by sequential use of agents such as 17% Ethylenediaminetetraacetic Acid (EDTA) to ensure comprehensive canal cleanliness. Another challenge lies in their narrow host range monophage preparations may be ineffective in polymicrobial infections. This issue can be mitigated through the use of bacteriophage cocktails specifically tailored to target multiple pathogenic species implicated in endodontic infections.

Conclusion

This study highlights the effectiveness of bacteriophages in eliminating *Enterococcus faecalis* biofilms within root canals, demonstrating their potential as a novel therapeutic option in endodontics. In addition to their targeted antibacterial action, bacteriophages offer advantages such as immune modulation, biofilm penetration and preservation of the oral microbiota. Their biocompatibility and ability to self-replicate make them a promising alternative or adjunct to traditional disinfectants, especially amid growing antibiotic resistance. Further research is needed to validate these findings and establish clinical protocols for their use in endodontic practice.

Conflict of Interest

The authors have declared no conflict of interest.

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