IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 24, Issue 9 Ser. 4 (September. 2025), PP 45-51
www.iosrjournals.org

" Lytic Bacteriophages Against Enterococcus Faecalis As An Alternative For Endodontic Infections"

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Abstract
Aim: *To detect bacteriophages with lytic activity against Enterococcus faecalis of endodontic origin.*
Background: *Phage therapy is a promising tool for developing innovative therapeutic strategies against persistent infections caused by Enterococcus faecalis in the oral cavity. Bacteriophages offer significant advantages over conventional antimicrobials, including their high specificity for bacterial strains, their host-dependent self-limiting replication capacity, and their proven safety during treatment, as they do not affect eukaryotic cells.*
Materials and Methods: *Detection of lytic phages using the double agar layer technique, using Enterococcus faecalis isolates of endodontic origin as hosts and wastewater samples as a source of bacteriophages. The isolated phages' characterisation was according to the morphology of the lysis plaques, the range of hosts, and their stability against pH and temperature variations.*
Results: *Two phages, EfaEnd1 and EfaEnd2, with specific lytic activity against endodontic E. faecalis were isolated. These phages showed stability at pH 7.0 ± 0.2 and temperatures up to 60 ± 2 °C. Their specificity and stability suggest potential therapeutic use against resistant and virulent strains.*
Conclusion: *The isolated lytic phages demonstrated high specificity and stability, making them a promising alternative for controlling resistant E. faecalis in endodontic infections. Their use could complement conventional treatments, reducing bacterial resistance. Further studies are needed to evaluate their efficacy "In Vivo".*
Keywords: *Enterococcus faecalis, bacteriophages, phage therapy*

Date of Submission: 09-09-2025 Date of Acceptance: 19-09-2025

I. Introduction

Persistent endodontic infections are a significant therapeutic challenge in dental practice, being one of the principal causes of root canal treatment failure. [1, 2] Among the microorganisms primarily involved in endodontic treatment failure is *Enterococcus faecalis*, a Gram-positive coccus characterised by its intrinsic and acquired resistance to multiple antibiotics and whose genetic plasticity allows it to adapt to adverse environmental conditions, making it difficult to eradicate using conventional therapeutic protocols. [3] *Enterococcus faecalis* is capable of surviving in hostile microenvironments characteristic of the root canal system, tolerating high temperatures, high salt concentrations, variable pH levels, and severe nutrient limitations. Additionally, it expresses various virulence factors, forms complex biofilms, evades the host's immune response, and is resistant to chemical and mechanical disinfection procedures, including irrigants and intracanal medications. [3, 4, 5, 6, 7]

Given the limitations of conventional intracanal disinfection methods and the growing problem of antimicrobial resistance in oral strains of *Enterococcus faecalis* [8, 9], phage therapy is a promising therapeutic alternative. In particular, lytic bacteriophages have promising characteristics against persistent endodontic infections, due to their ability to penetrate biofilms, replicate in situ without integrating into the host DNA, lyse bacterial strains including those resistant to antibiotics, and perform horizontal gene transfer without affecting the host's eukaryotic cells. [10, 11]

Preliminary studies have demonstrated the efficacy of specific lytic phages against *Enterococcus faecalis* in "*In Vitro*" and "*Ex Vivo*" models, through the reduction of bacterial load, destabilisation of biofilms, and lysis of both antibiotic-sensitive and antibiotic-resistant isolates. [12, 13, 14] However, to achieve effective clinical implementation of phage therapy in the endodontic context, it is essential to consider the high phenotypic diversity of both bacteriophages and their hosts. [15, 16] Consequently, before therapeutic application, it is crucial to understand in detail the interaction between the phage and the host, as well as the relationship with extrinsic factors associated with endodontic treatments. Physicochemical conditions, such as pH, temperature, compatibility with irrigation solutions, and the filling materials, can be altered by the clinical environment, which directly impacts the stability of the phage, its adsorption capacity and the dynamics of bacterial growth. [17, 18] This information highlights the need to continue searching for lytic phages whose specificity and stability are not compromised by physical-chemical variations in the environment, to develop a bioproduct that, when applied clinically, increases the success rate of conventional endodontic treatment. This product would reduce the need for endodontic retreatment, whether orthograde or retrograde, as these contribute to the progressive weakening of the dentinal structure and increase the risk of vertical fractures and the consequent tooth loss. In this context, the present study aimed to isolate phages from wastewater and evaluate their lytic capacity against clinical isolates of *Enterococcus faecalis*.

II. Material And Methods

Wastewater samples

Wastewater samples were from the effluents of a municipal treatment plant, including randomly selected points with high faecal contamination loads, as well as points corresponding to different stages of water treatment. The collection occurs by following the methodological guidelines established in the 24th edition of Standard Methods for the Examination of Water and Wastewater. [19] To obtain the phage lysate, the supernatants were filtered, purified, concentrated and stored according to the protocol described above. [20]

Detection of bacteriophages

The detection of phages with lytic activity was evaluated against 14 isolates of *Enterococcus faecalis* of endodontic origin, previously identified using the Dade/MicroScan Pos ID PC34 system (West Sacramento, CA, USA) and the MALDI-TOF MS Biotyper mass spectrometry system (Bruker Daltonics, Germany), with reference to the Bruker Taxonomy Database Version 3.3.1 in accordance with previous guidelines. [3, 7] The modified Double-Layer Agar method (DLA) served, using 1.5% (w/v) Cromocult Enterococci agar in the lower layer and 0.5% (w/v) in the upper layer. To each 3 mL of soft agar (45 ± 2 °C), 100 µL of bacterial culture in the logarithmic growth phase was added. The mixture was poured onto Petri dishes with already solidified base agar and, after solidification, 20 µL of phage filtrate was inoculated and allowed to absorb at 20 ± 2 °C with subsequent incubation between 24 and 48 hours at 37 °C. [21, 22] The presence of translucent lysis plaques or inhibition zones was interpreted as indicative of lytic activity. [23] Lysis plaque-forming units (PFU/mL) were calculated considering the dilution of the sample, according to standard criteria and controls used to corroborate the specificity of the assay. [23, 24] Plates with defined lysis zones were purified, concentrated, and the isolated phages preserved according to standard guidelines established for the double-layer agar method. [25] The assays were performed in triplicate.

Phagues characterisation

Phenotypic characteristics

The lysis plates morphology was observed and recorded based on the halo, size, texture, turbidity and edges, selecting those that had clearly defined lytic zones. [26]

Determination of host range

As an initial screening, control strains *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecalis* ATCC 700802 served to determine the spectrum of action of the phages against different bacterial genera present in the oral cavity; the reference strains *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Streptococcus mutans* ATCC 25175, and *Leptotrichia buccalis* ATCC 14201 served to determine the spectrum of action of the phages against different bacterial genera present in the oral cavity. Cultures were incubated in BHI broth with constant agitation until they reached the logarithmic growth phase. [3, 7] The host range was classified as specific, moderate and broad according to pre-established parameters. [27]

pH and temperature stability

The phage thermal stability and pH tolerance assays were performed randomly after selecting three points considered critical for its replication (low, medium and high). The tests employed the DLA methodology, with an initial concentration of 10^6 PFU/mL. The phages were incubated in BHI broth for 1 hour at temperatures

of 10, 37, and 60 ± 2 °C, pH of 3, 7, and 11 ± 0.2, respectively. The collection of samples occurred every 10 minutes. [28]

Data analysis

The research corresponded to an initial qualitative and descriptive pilot study, in which no statistical models were applied.

III. Results

Characteristics of** Enterococcus faecalis **isolates

Fourteen *Enterococcus faecalis* isolates of endodontic origin were selected for convenience, which simultaneously acquired resistance to at least one of the following antibiotics: erythromycin, nitrofurantoin, ciprofloxacin, gentamicin or high-level streptomycin, and presented virulence genes associated with biofilm formation.

Isolation of bacteriophages

Phages recovered from sampling points with the highest probability of human and animal faecal contamination. During this study, a total of 10 different phages resulted from wastewater infection of the ATCC control strains, of which two have clearly defined characteristics of lytic activity.

Phenotypic characteristics

Morphological characteristics were observed: eight phages presented opaque plaques or central bacterial development in the form of a bull's-eye corresponding to a lysogenic pattern, and two phages, named EfaEnd1 and EfaEnd2, presented PFUs with an average of 2 ± 1 mm, translucent, with defined edges compatible with a classic lytic pattern.

Evaluation of host range

The evaluation of host range allowed the isolated phages to be classified as specific, since the ATCC reference strains, belonging to different bacterial genera and species other than *Enterococcus faecalis*, were not susceptible to their lytic action (Table 1). This lack of tropism remained constant both when the phages were evaluated individually and in phage cocktails. Furthermore, the formulation of these cocktails did not result in an expansion of the lysis spectrum, confirming the specificity of the phages towards *E. faecalis* as their exclusive host.

When analysing the lytic activity on clinical strains of *E. faecalis* of endodontic origin, EfaEnd1 phage was capable of lysing 64% (9/14) of the strains evaluated, while EfaEnd2 showed lytic activity on 36% (5/14) of them.

Table 1. Phages' activity against different bacterial genera and species.

Bacterial species	EfaEndo 1	EfaEndo 2
E. faecalis	(+) 64 %	(+) 36 %
S. aures	(-)	(-)
S. mutans	(-)	(-)
E. coli	(-)	(-)
L. bucalis	(-)	(-)

(+) remarkable phage activity, (−) no phage activity.

pH and temperature stability 5/13.

The viability of the phages remained constant at pH 7.0 ± 0.2, while a marked reduction occurred under extreme pH conditions. In particular, a 70–80% decrease in viability happened at pH 3.0 ± 0.2, and approximately 20–25% at pH 11.0 ± 0.2 (Figure 1).

Similarly, thermal stability analysis revealed that the phages retained 100% of their viability at 10°C. However, stability decreased progressively with increasing temperature, reaching a reduction of 15–20% at 37 ± 2 °C and a more pronounced loss of 40–60% at 60 ± 2 °C (Figure 1).

The EfaEnd1 phage had slightly higher stability to changes compared to the EfaEnd2 phage.

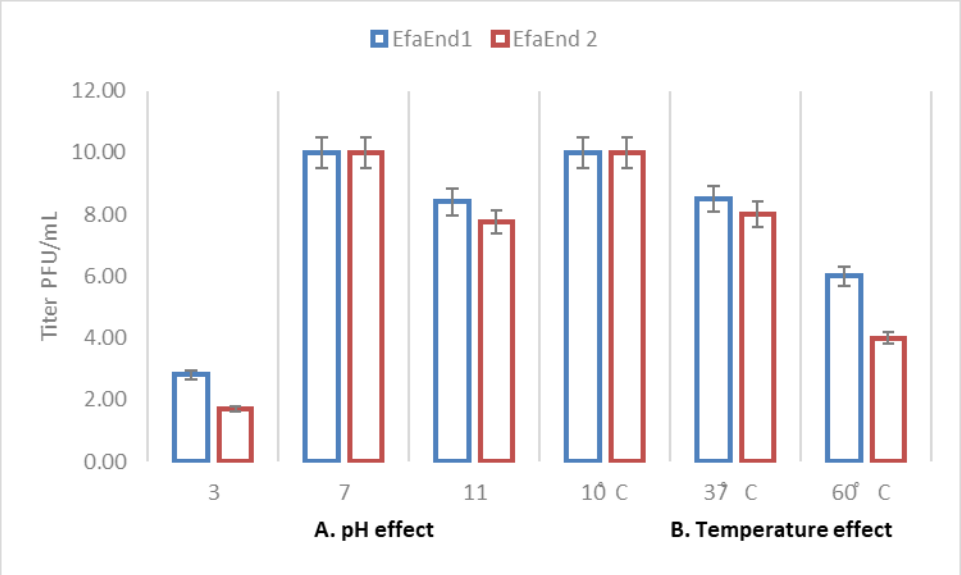

Figure 1. Thermal and pH stability of phages EfaEnd1 and EfaEnd2. Left: Infectivity after exposure to different pH values (3.0, 7.0, and 11.0 ± 0.2). Right: Infectivity after exposure to three temperature points (10, 37, and 60 ± 2 °C) for 1 hour. Results expressed as the percentage of the initial phage titer (PFU/mL) retained after treatment. Data represent the mean ± standard deviation of three independent experiments.

IV. Discussion

In this study, clinical isolates of *Enterococcus faecalis* of endodontic origin serve as hosts, which presented patterns of acquired resistance to multiple antibiotics, as well as the presence of virulence genes related to biofilm formation. The selection of these characteristics is particularly relevant in the context of dental practice, as *E. faecalis* is a pathogen associated with persistent endodontic infections, whose ability to form biofilms and resist antimicrobial treatments represents an increasing therapeutic challenge. [29]

Faced with this problem, the development of alternative therapeutic strategies, such as phage therapy, has gained growing interest. Due to phage bacterial specificity and ability to replicate at the site of infection, they are emerging as a novel and promising approach to controlling resistant and virulent intraradicular bacteria. [30] Several recent studies have evaluated the use of phage therapy in in vitro, "*In Vivo*", and "*Ex Vivo*" models, demonstrating not only its antimicrobial utility but also its benefits in terms of specificity, safety profile, and cost-effectiveness. [31, 32, 33, 34]

However, despite these advances, the percentage of phages currently used in clinical therapies remains very low [35]; this is because many questions remain about their application, including: the possible emergence of mutations in bacterial receptors leading to phage resistance [36], synergistic or antagonistic interactions with conventional antibiotics [37], side effects on commensal microbiota [38, 39]; stability in replication processes [8], as well as the infective efficacy of phages under variable pH and temperature conditions, factors that are particularly relevant in the endodontic environment [16], some of which were in this study assayed.

Wastewater is a rich and accessible source for isolating lytic phages active against *Enterococcus faecalis*, including those with antibiofilm capacity and therapeutic potential in persistent endodontic infections. In this study, out of a total of 10 phages obtained from sources with a high probability of human and animal faecal contamination, two phages (EfaEnd1 and EfaEnd2) were isolated that showed characteristics compatible with a lytic cycle. This finding is particularly relevant, as phages with a strict lytic cycle are preferred for therapeutic applications because they do not integrate their genetic material into the host genome, causing the immediate lysis of the bacterial cell. [14, 30]

The phages isolated in this study demonstrated high specificity against *Enterococcus faecalis*, as well as stability across wide pH and temperature ranges, reinforcing their potential to replicate in the adverse conditions of the endodontic environment. [16, 30] This specificity is clinically relevant, as it suggests that the phages recognise highly conserved receptor structures unique to *E. faecalis*. [33]

However, the results revealed that only between 36 and 64% of the strains were susceptible to the phages evaluated, indicating that the phages do not have a broad enough host range to recognise all strains of *E. faecalis*. This phenomenon can be due to several biological factors that limit the host range; one of the main factors is the variability in bacterial surface receptors. In the case of *E. faecalis*, the enterococcal polysaccharide antigen (EPA) acts as a key receptor for many phages; however, it can undergo mutations or modifications in both its core genes and the variable genes of the epa locus, which prevents efficient adsorption [40, 41]. Additionally, even if adsorption

occurs, productive infection is not always guaranteed. In some cases, phages fail to inject their genetic material or cannot replicate due to incompatibilities with host defence mechanisms, such as restriction-modification systems, CRISPR-Cas or abortive responses. [42] Additionally, this is the high genetic plasticity of *E. faecalis*, a species that exhibits remarkable phenotypic diversity between strains, with structural variations in capsules and cell wall components that affect interaction with phages. [3, 10, 14] Finally, it has been documented that under selective pressure exerted by phages, bacteria can evolve evasion mechanisms, such as mutations in receptors or negative regulation of their expression, which generate resistance to the phage. [33, 36] Although this specificity is an advantage in terms of selectivity and protection of the resident microbiota, it also represents a limitation when it comes to polymicrobial infections. In fact, recent studies suggest that *E. faecalis* persist not only because of its own virulence factors, but also due to synergistic interaction with metabolites from other microbial species present in the biofilm. [32, 38] For these reasons, phage therapy studies should focus on identifying the genetic determinants that condition the host range. For the effectiveness of clinical application, especially in the endodontic setting, it is essential to perform molecular characterisation of circulating strains to select highly compatible phages with proven efficacy against prevalent clones.

In terms of their physicochemical stability, the lytic phages have promising replicative growth at pH 7.0 ± 0.2 and temperatures near 37 ± 2°C, conditions that coincide with the physiological microenvironment of the oral cavity. This finding is relevant from a therapeutic perspective, as it suggests phage viability in real clinical settings. This study detected a progressive decrease in phage infectivity at extreme pH or high temperature. At basic pH, lytic activity decreased by 25%, at acidic pH it decreased by 80%, while at temperatures above 60 ± 2 °C it decreased by up to 60% (figure 1). This loss of biological activity is due to the denaturation of the structural capsid proteins, which are essential for maintaining the integrity of the virion and protecting the viral genetic material. These proteins are sensitive to thermal destabilisation and changes in the ionisation of their functional groups, especially in highly acidic or alkaline environments. At temperatures above 60 ± 2 °C, the capsid loses its three-dimensional conformation, compromising DNA encapsulation and, therefore, its ability to infect. Similarly, pH values < 4.0 ± 0.2 or > 10.0 ± 0.2 can modify the surface charge of the virion, affecting its interaction with bacterial receptors and promoting the degradation of the viral genome. [16, 30, 43]

Understanding these conditions is crucial for proposing the use of phages as therapeutic agents, given that their activity can be significantly affected by variations in pH and temperature. During the root canal filling process, commonly used materials such as $Ca(OH)_2$ (calcium hydroxide) and certain endodontic cements generate a local microenvironment with a high alkaline pH (pH > 11± 0.2) due to the release of hydroxyl ions (OH^-). In addition, the chemical reactions associated with polymerisation can produce local thermal increases, reaching temperatures of up to 50 ± 2°C, conditions that can compromise the stability and efficacy of phage lysis. On the other hand, in contexts of chronic inflammation or persistent infections, the pH can become acidic as a consequence of the accumulation of bacterial metabolites and the inflammatory response, creating an environment that is equally adverse to phage viability and its therapeutic potential. [44, 45] Despite current limitations, the discovery of the EfaEnd1 and EfaEnd2 phages provides a promising basis for future research to complete their molecular characterisation, validate their efficacy in relevant clinical contexts, and explore their synergistic potential with antibiotics and their ability to destroy biofilms.

V. Conclusion

The results obtained demonstrate that the isolated lytic bacteriophages exhibit high specificity against strains of *Enterococcus faecalis* of endodontic origin, characterised by antimicrobial resistance profiles and virulence factors related to biofilm formation. The stability observed under pH and temperature conditions compatible with the endodontic environment suggests that these phages could maintain their viability during clinical application. The absence of lytic activity against other bacterial species indicates a low risk of altering the normal microbiota, reinforcing their potential as a selective therapeutic tool. Although the study is at a preliminary stage, these findings open up a promising avenue for the development of phage therapies as adjuvants or alternatives to conventional treatment, especially in persistent or recurrent infections.

Declarations

Ethical approval: The study did not involve direct research with humans or animals and was approved by the Institutional Ethics Committee of the Faculties of Science and Dentistry..

Acknowledgements: We thank the Pontificia Universidad Javeriana for the institutional, technical, and technological support provided through project ID: 00007914 "*Caracterización de Enterococcus faecalis de origen endodóntico: perfiles de susceptibilidad antibiótica, expresión de factores de virulencia y actividad ante fagos líticos*" y al Dr. Raúl A. Poutou-Piñales, Ph.D. for English editing.

Founding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions: All authors contributed significantly to the work, from the design and execution of the study to the analysis and interpretation of data. They participated in the writing and critical review of the article. They approved the final version for publication and chose the journal for submission.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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