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Synergistic Interaction Between Lactoferrin and Low Dose Antibiotic Against Fusobacterium Nucleatum Associated with Periodontal Disease

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Abstract

Background

Periodontitis is a chronic inflammatory disease characterized by the progressive destruction of periodontal tissues, significantly impacting oral and systemic health. Fusobacterium nucleatum (Fn) plays a key role in disease pathogenesis due to its interactions with other periodontal pathogens. Lactoferrin (LF), a multifunctional glycoprotein, has demonstrated antimicrobial and anti-inflammatory properties, but its effects in combination with antibiotics against Fn are not fully understood.

Objective

To evaluate the antibacterial activity of bovine lactoferrin (bLF), in combination with low concentrations of penicillin-streptomycin, against F. nucleatum, and to assess its potential as an adjunctive therapeutic strategy in periodontitis.

Methods

Bovine lactoferrin (bLF) was prepared at a concentration of 20 mg/mL in sterile distilled water. The dual antibiotic solution (penicillin–streptomycin) was adjusted to a final concentration of 0.604 µg/mL. For the combination treatment, 10 µL of the antibiotic solution was added to 1 mL of the bLF solution, yielding a final antibiotic concentration of approximately 0.003 µg/mL. This combination, along with antibiotic-only and control groups, was tested in vitro against Fusobacterium nucleatum (ATCC 25586) cultured under anaerobic conditions at 37 °C. Bacterial growth was assessed by optical density (OD_{600}) measurements and colony-forming unit (CFU) enumeration. Controls included bacteria without lactoferrin or antibiotics.

Results

Lactoferrin significantly inhibited bacterial growth in a dose-dependent manner. Low-concentration antibiotics alone had negligible antibacterial effects, with bacterial growth comparable to controls ($p > 0.05$). The combination of lactoferrin with antibiotics enhanced bacterial inhibition, indicating a synergistic effect.

Conclusions

Bovine lactoferrin, particularly in combination with sub-therapeutic antibiotic concentrations, shows promising antibacterial activity against F. nucleatum, suggesting its potential as an adjunctive agent in periodontitis management. These findings warrant further in vivo studies to confirm efficacy and explore clinical applications.

Keywords: Lactoferrin, Structure, Biological Functions, and Potential In Periodontal Disease Treatment

1. Introduction

Periodontitis is a prevalent chronic inflammatory disease characterized by the progressive destruction of periodontal tissues, including the gingiva, periodontal ligament, and alveolar bone. If left untreated, periodontitis can lead to tooth loss, significantly impairing both oral health and quality of life. This multifactorial disease is primarily driven by microbial dysbiosis, which plays a pivotal role in its pathogenesis. Key microbial species, such as Fusobacterium nucleatum, are critical members of the oral microbiome, contributing to the development and progression of periodontal diseas. Understanding these pathogens in greater detail is essential

for the prevention and effective treatment of periodontitis [1].

In addition to their association with periodontal disease, these oral pathogens have been implicated in various systemic conditions, including gastrointestinal disorders such as colorectal cancer and inflammatory bowel disease, cardiovascular diseases, immune-mediated disorders like rheumatoid arthritis, as well as respiratory diseases and neurological conditions such as Alzheimer's disease. The management of periodontitis is a multistage process that involves the elimination of etiological factors, control of inflammation, restoration of periodontal health, and prevention of disease recurrence. The primary therapeutic strategy relies on mechanical debridement to remove local irritants, such as calculus and plaque biofilm. Despite the partial success of current therapies in controlling inflammation and promoting tissue repair, significant challenges remain. Notably, prolonged use of antibiotics in periodontal therapy can lead to bacterial resistance and microbial dysbiosis, thereby compromising therapeutic efficacy and increasing the complexity, cost, and failure rates of treatment [2-5].

Given the persistent limitations of conventional periodontal therapies particularly the risks of microbial dysbiosis and antibiotic resistance there is a growing imperative to investigate novel adjunctive treatment strategies. Among emerging candidates, lactoferrin, a multifunctional glycoprotein with well-documented antimicrobial and immunomodulatory activities, has shown considerable potential in enhancing therapeutic outcomes. When used alongside low-dose antibiotics, lactoferrin may exert synergistic effects. Such adjunctive applications could significantly reduce reliance on high-dose or prolonged antibiotic regimens, thereby minimizing associated adverse effects. This study aims to evaluate the efficacy of lactoferrin as an adjuvant to low-dose antibiotic therapy in the management of periodontal disease, with a specific focus on its inhibitory impact on Fusobacterium nucleatum, a key pathogen in periodontal pathogenesis. The investigation seeks to contribute to the development of integrated, microbiome-friendly therapeutic approaches that offer improved clinical and microbiological outcomes.

2. Structural Characteristics

Lactoferrin (LF) is an 80 kDa non-heme iron-binding glycoprotein that belongs to the transferrin family. It is widely distributed in mammalian secretions, including saliva, milk, tears, and gastrointestinal fluids. Additionally, lactoferrin is released by neutrophils during immune responses, where it contributes significantly to host defense. Within the oral cavity, lactoferrin plays a crucial role in mucosal protection, being present in both saliva and gingival crevicular fluid, thereby contributing to the prevention of pathogen colonization in periodontal tissues. Structurally, lactoferrin comprises two homologous lobes, the N-lobe and the C-lobe, each of which possesses a high-affinity binding site for ferric ions (Fe^{3+})[5]. This iron-binding capacity is central to its antimicrobial function, as it limits bacterial growth by sequestering iron, an essential nutrient for microbial proliferation, as illustrated in Figure 1 [6,7].

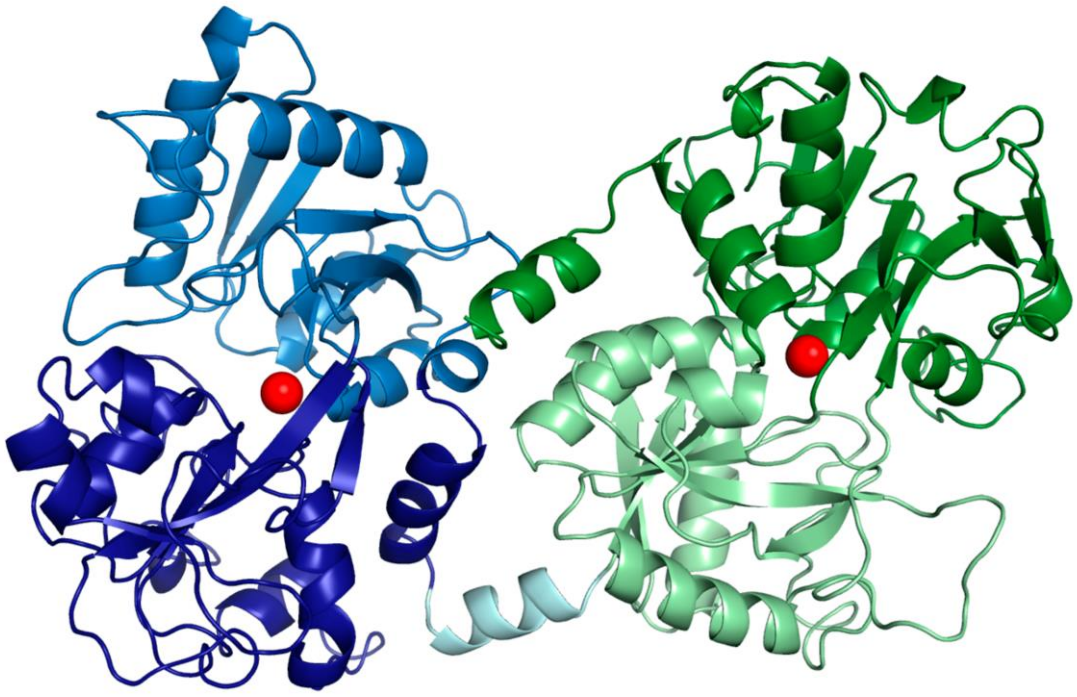

Figure 1: Three-Dimensional Structure Of Lactoferrin [8]

2.2. Biological Functions of Lactoferrin

2.2.1. Antimicrobial Activity

Lactoferrin exerts both bacteriostatic and bactericidal effects through multiple mechanisms. Its high-affinity iron-binding capacity deprives bacteria of this essential nutrient, with iron sequestration being particularly effective under acidic conditions typical of periodontal infections. In addition, lactoferrin modulates the host immune response by stimulating cytokine and chemokine production, enhancing phagocytic activity, and promoting bacterial clearance. The cationic N-terminal region of lactoferrin disrupts bacterial membranes, increasing permeability and causing cell lysis, while proteolytic cleavage generates antimicrobial peptides, that target bacterial membranes and lipopolysaccharide [9-11].

2.2.2. Potential Synergy with Antibiotics

One of the key advantages of lactoferrin in periodontal therapy is its potential synergy with low-dose antibiotics. Lactoferrin's iron-chelating properties deprive bacteria of essential nutrients, while its ability to disrupt bacterial membranes enhances the activity of antibiotics. This synergy could significantly enhance the efficacy of antibiotics, even at sub-inhibitory concentrations, reducing the risks of antibiotic resistance and microbial dysbiosis, which are common problems in long-term antibiotic therapy [4- 13].

2.3. Therapeutic Applications of Lactoferrin In Oral and Systemic Health

Lactoferrin (LF) demonstrates remarkable therapeutic versatility across multiple biological systems, highlighting its potential as an adjunctive agent in managing oral and systemic infections. For instance, LF has shown significant efficacy against Candida-associated denture stomatitis by inhibiting biofilm formation and enhancing the activity of conventional antifungal agents such as azoles and polyenes. Beyond its antifungal role, LF when combined with lactoperoxidase (LPO) has been clinically validated to improve oral hygiene outcomes by reducing volatile sulfur compounds and suppressing Porphyromonas gingivalis levels without disrupting commensal microbiota. Additionally, its integration into photodynamic therapy protocols has demonstrated promising antifungal outcomes against multidrug-resistant Candida species, particularly when activated by both hydrogen peroxide and light exposure. Beyond oral health, LF exhibits notable anticancer effects, selectively targeting malignant cells while sparing normal tissues through modulation of pathways governing cell proliferation, apoptosis, migration, and immune activation. Furthermore, its broad-spectrum antiviral propertie mediated through receptor competition, direct viral binding, and immune modulation underscore its multifunctional nature in host defense . Collectively, these findings position lactoferrin as a bioactive molecule with diverse biomedical applications and establish a strong foundation for exploring its synergistic antibacterial activity alongside low-dose antibiotics, particularly in infections involving Fusobacterium nucleatum and other periodontal pathogens [14- 17].

3. Experimental Design

3.1. Materials

- **Bovine Lactoferrin (BLF):** Obtained commercially and dissolved in sterile distilled water to prepare stock solutions.
- **Fusobacterium Nucleatum (ATCC 25586):** Cultivated in Brain Heart Infusion (BHI) agar and broth under anaerobic conditions at 37°C.
- **Antibiotic Preparation:** A penicillin-streptomycin (Pen-Strep) solution was prepared at a final concentration of 0.604 µg/mL.

3.2. Preparation of Experimental Solutions

Three separate tubes were prepared for the experimental treatments. Tube 1 contained the dual antibiotic solution (penicillin-streptomycin) at a concentration of 0.604 g/mL. Tube 2 contained lactoferrin at a concentration of 20 mg/mL, to which 10 µL of the antibiotic solution from Tube 1 was added, resulting in a final antibiotic concentration of approximately 0.003 µg/mL. Tube 3 contained 1 mL of sterile saline solution supplemented with 10 µL of the antibiotic solution from Tube 1, also resulting in a final antibiotic concentration of approximately 0.003 µg/mL. All solutions were prepared under sterile conditions and mixed thoroughly to ensure homogeneity prior to use in the experiments.

3.3. Antibacterial Assay

The antibacterial activity of lactoferrin was evaluated using two 96-well microdilution assays. Bacterial growth was assessed by measuring optical density at 600 nm (OD_{600}) and enumerating colony-forming units (CFUs). For the first 96-well plate, 50 µL of the lactoferrin solution from Tube 2 (20 mg/mL with 10 µL of dual antibiotics) was combined with 50 µL of F. nucleatum culture in BHI medium, yielding a final actoferrin concentration of 10 mg/mL in the first well. To obtain lower concentrations, 50 µL from the first well was transferred to the second well containing 50 µL of bacterial culture, resulting in a concentration of 5 mg/mL. The procedure was repeated to achieve a third concentration of 2.5 mg/mL. The last column served as a control without antibiotics or lactoferrin. For the second 96-well plate, 50 µL of the antibiotic-diluted solution from Tube 3 was added to 50 µL of bacterial culture in BHI medium at three different concentrations. A control row without lactoferrin or antibiotics was also prepared. Both plates were incubated under appropriate conditions for 48 hours. After incubation, bacterial growth was quantified by OD_{600} measurement, and CFUs were enumerated for each concentration in the second plate.

3.4 Statistical analysis

Statistical analyses were performed using SPSS version 26 (IBM Corp., Armonk, NY). Data are presented as mean ± standard deviation (SD). Intergroup differences were evaluated using one-way analysis of variance (ANOVA).

4. Results

4.1 Antibacterial Activity of Lactoferrin

The combination of lactoferrin with dual antibiotics

(penicillin-streptomycin) exhibited significant antibacterial activity against Fusobacterium nucleatum, as evidenced by a marked reduction in bacterial growth compared to the control group that received no treatment ($p < 0.05$). The optical density (OD_{600}) measurements taken after 48 hours of incubation demonstrated that the combined treatment substantially inhibited bacterial proliferation, confirming the synergistic antibacterial effect of lactoferrin and antibiotics, as shown in Figure 2 and table 1.

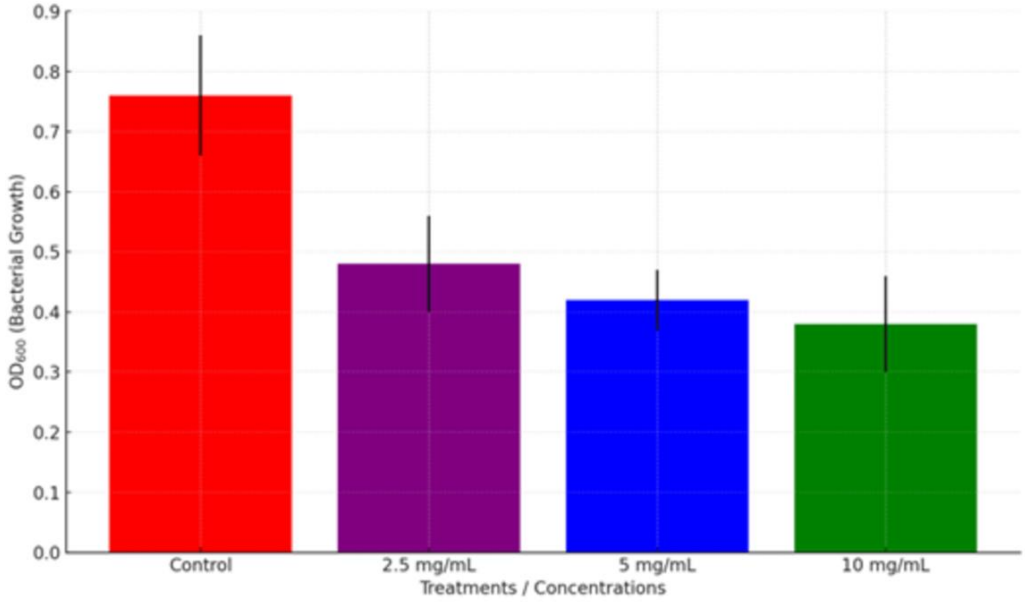

Figure 2: Effect of BLF on The Growth of Fusobacterium Nucleatum, Represented by Average Optical Density (OD600) After Treatment with Different Concentrations Of BLF

Concn (mg/mL)	2.5	5	10	Control
First value	0.395	0.416	0.353	0.842
Second value	0.505	0.455	0.308	0.800
Third value	0.544	0.370	0.469	0.636

Table 1: Optical Density (OD_{600}) Measurements of Fusobacterium Nucleatum at Different Concentrations of Lactofferin

In addition to optical density measurements, colony-forming unit (CFU) enumeration further corroborated the antibacterial effect, with a corresponding decrease in CFUs as the concentration of lactoferrin increased, highlighting a dose-dependent response (Figure 3). This suggests that lactoferrin, when combined with antibiotics, can effectively suppress *F. nucleatum* growth, particularly at higher concentrations of lactoferrin.

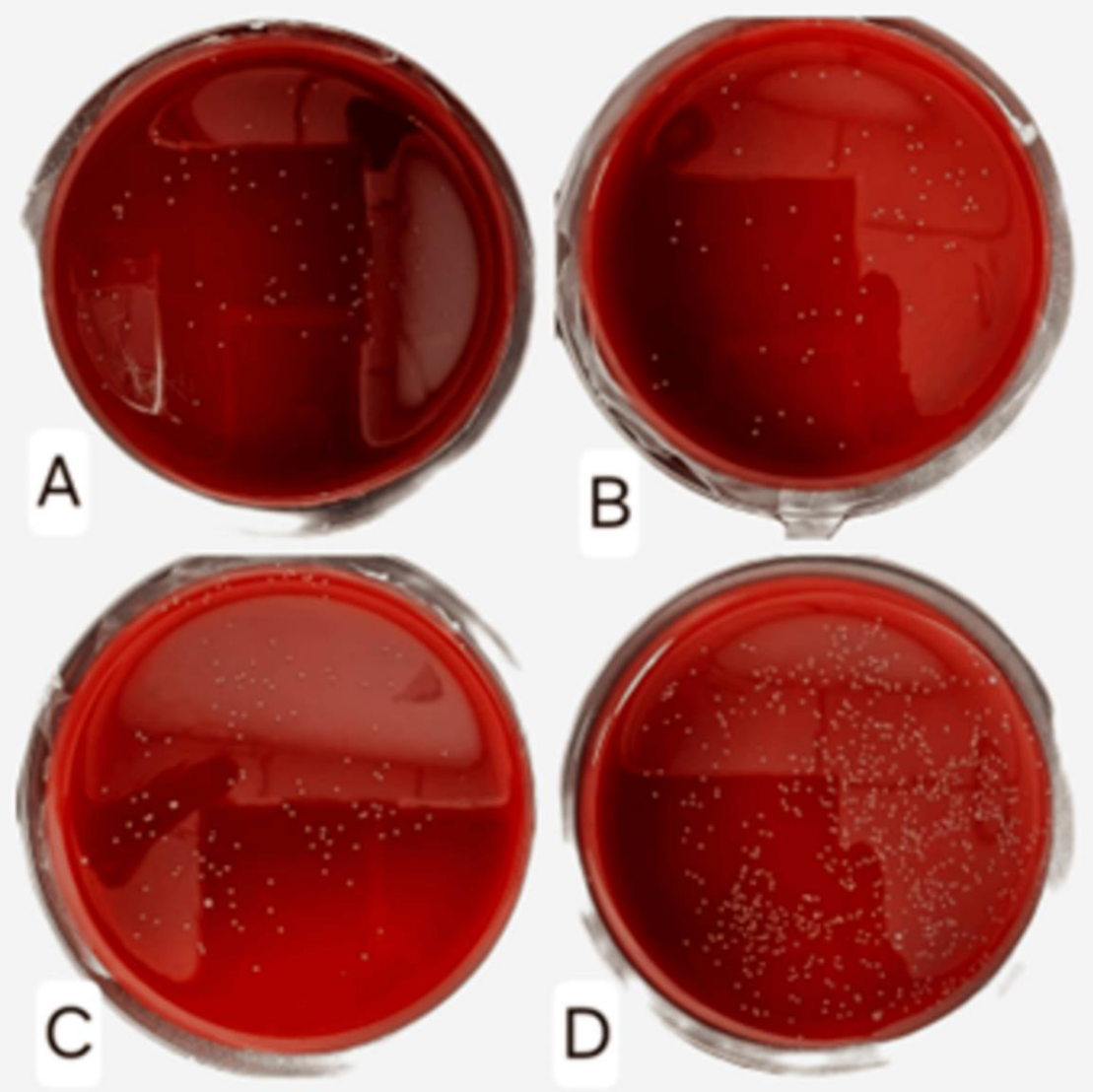

Figure 3: CFUS of Fusobacterium Nucleatum after Treatment with Different Concentrations of Bovine Lactoferrin

Concentration of bLf is 10 mg/mL. B: Concentration of bLf is 5 mg/mL. C: Concentration of bLf is 2.5 mg/mL. D: Concentration of bLf is 0 mg/mL (control, no lactoferrin added). Exposure of Fusobacterium nucleatum to low concentrations of dual antibiotics demonstrated minimal antibacterial activity. The optical density measurements after 48 hours revealed that bacterial growth patterns at these low antibiotic concentrations were nearly identical to those observed in the untreated control (mean OD_{600}: 0.785–0.827 vs. control 0.895). This suggests that the sub-inhibitory concentrations of the antibiotics, when tested alone, were ineffective in inhibiting bacterial growth. These results were further supported by statistical analysis using one-way ANOVA, which confirmed the absence of significant differences among the antibiotic-treated groups and the control ($p > 0.05$). Thus, it is evident that, at the concentrations tested, the antibiotics alone did not exhibit significant antibacterial activity against F. nucleatum. In summary, while lactoferrin demonstrated a strong antibacterial effect in combination with antibiotics, the low antibiotic concentrations tested alone had little to no effect on bacterial growth. This reinforces the idea that the combination of lactoferrin and antibiotics can provide enhanced antibacterial efficacy, while low antibiotic concentrations may not be effective in the absence of lactoferrin, as shown in Figure 4 and table 2.

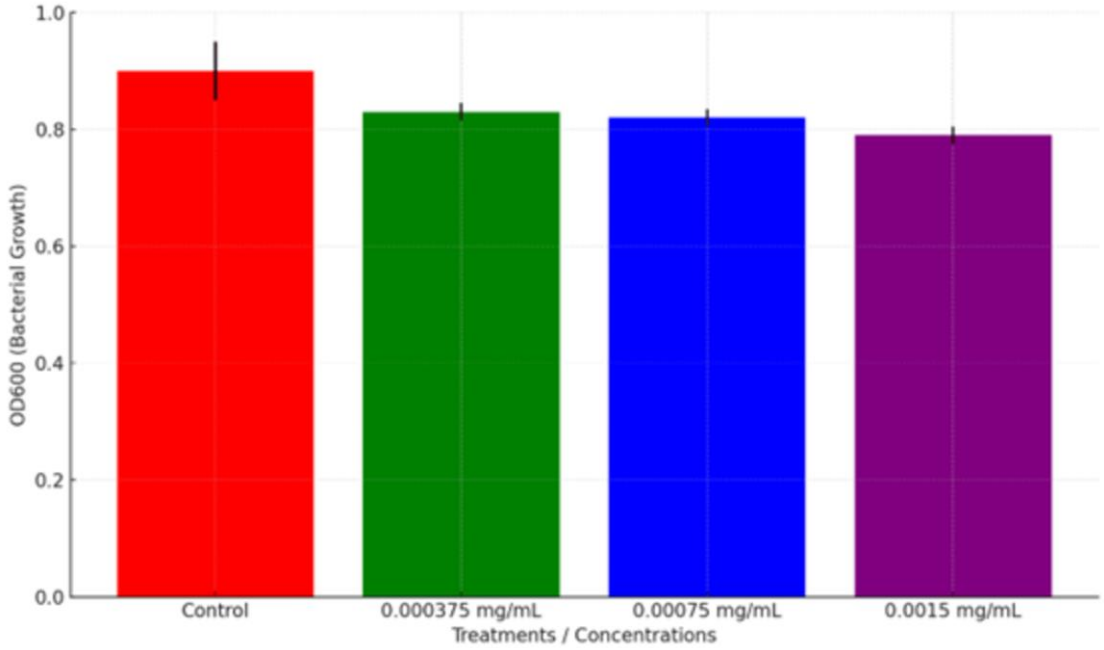

Figure 4: Low Dose Dual Antibiotic Effects on Bacterial Growth

Concn (µg/mL)	0.000375	0.00075	0.0015	Control
First value	0.839	0.843	0.778	0.844
Second value	0.789	0.774	0.816	0.832
Third value	0.853	0.828	0.761	1.008

Table 2: Optical Density (OD_{600}) Measurements of Fusobacterium Nucleatum at Different Concentrations of Penicillin-Streptomycin

5. Discussion

The antibacterial activity of lactoferrin was evaluated along and in combination with a dual antibiotic solution (penicillin-streptomycin) against Fusobacterium nucleatum. The results demonstrated that lactoferrin exhibited a clear inhibitory effect on bacterial growth, which was further enhanced when combined with the dual antibiotics at the tested concentrations. Specifically, the combination of lactoferrin (10 mg/mL) and the diluted antibiotics (approximately 0.0015 µg/mL) significantly reduced bacterial growth, as evidenced by decreased OD_{600} values and CFU enumeration, compared to controls without treatment. Statistical analysis (one-way ANOVA) confirmed that these differences were significant, highlighting the synergistic effect of lactoferrin and antibiotics at these concentrations. Conversely, the dual antibiotics alone, when diluted to very low concentrations, had minimal or no observable effect on bacterial growth. OD_{600} measurements across the different concentrations were nearly identical and comparable to the untreated control, indicating that the bacterial cells were largely unaffected by the low antibiotic concentrations. These findings suggest that, under the experimental conditions used, the low-dose antibiotic solution is insufficient to inhibit F. nucleatum growth, emphasizing the importance of proper dosing and the potential benefit of combining antibiotics with natural antimicrobial agents such as lactoferrin. The observed synergistic effect between lactoferrin and the dual antibiotics may be attributed to complementary mechanisms of action. Lactoferrin is known to disrupt bacterial membranes and sequester iron, thereby limiting bacterial proliferation, while penicillin-streptomycin interferes with cell wall synthesis and protein production. The combination likely enhances bacterial susceptibility, even at sub-inhibitory antibiotic concentrations. These results align with previous studies reporting the potentiation of antibiotic efficacy by lactoferrin and underscore its potential application as an adjunct antimicrobial agent. Importantly, lactoferrin is not only a direct antimicrobial agent but also a modulator of immune and inflammatory responses. It has been shown to regulate the production of pro-inflammatory cytokines, enhance the activity of immune cells such as neutrophils and macrophages, and reduce tissue damage associated with

bacterial infection. These immunomodulatory properties suggest that lactoferrin may provide a dual benefit: directly inhibiting bacterial growth while controlling inflammation, which is particularly relevant in periodontal and other oral infections where inflammation contributes to tissue destruction. Therefore, the synergistic effect observed in this study may reflect both direct antibacterial activity and indirect modulation of host immune defenses, supporting its potential as an adjunctive therapeutic agent [18-21].

Moreover, lactoferrin has been reported to prevent biofilm formation and disrupt pre-existing biofilms, which could further explain the potentiation of antibiotic activity observed in this study. Its ability to act on multiple bacterial targets while simultaneously modulating host responses suggests that lactoferrin could be particularly useful in overcoming antibiotic resistance, a growing concern among oral pathogens. In conclusion, the present study demonstrates that lactoferrin exhibits significant antibacterial activity against Fusobacterium nucleatum, and its combination with even low concentrations of penicillin-streptomycin can enhance this effect. However, the diluted antibiotics alone at very low concentrations showed negligible inhibitory activity, highlighting the importance of combinatorial treatment strategies. These findings suggest that lactoferrin may act synergistically with antibiotics, potentially allowing for reduced antibiotic dosages while maintaining antibacterial efficacy. Nevertheless, this study is limited by its in vitro design, which may not fully replicate the complex conditions of an in vivo environment, including host immune factors and microbial community interactions. Additionally, only a single bacterial strain and a narrow range of concentrations were tested, and the precise mechanisms underlying the observed synergistic effects remain unclear. Future investigations should expand to multiple bacterial strains, broader concentration ranges, and more physiologically relevant models, such as biofilm cultures or animal studies, to validate these results and explore the mechanistic basis of lactoferrin-antibiotic synergy. Overall, the current findings provide a promising basis for developing combinatorial antimicrobial strategies with potential clinical relevance [22].

6. Conclusion

This study demonstrates that lactoferrin exhibits significant antibacterial activity against Fusobacterium nucleatum. Its inhibitory effect was further enhanced when combined with penicillin-streptomycin, even at very low antibiotic concentrations. In contrast, the diluted antibiotics alone showed minimal to no effect on bacterial growth, underscoring the importance of combinatorial treatment strategies. The observed synergy likely arises from complementary mechanisms: lactoferrin's ability to disrupt bacterial membranes, sequester iron, modulate immune responses, and prevent biofilm formation, alongside the antibiotics' interference with cell wall synthesis and protein production. Together, these actions enhance bacterial susceptibility, suggesting that lactoferrin may reduce the effective antibiotic dose required while maintaining antibacterial efficacy. However, the study is limited by its in vitro design, the use of a single bacterial strain, and a narrow concentration range. Future research should include broader bacterial targets, physiologically relevant models, and mechanistic analyses to confirm and expand on these findings. Overall, lactoferrin holds strong potential as an adjunct antimicrobial agent, offering both direct antibacterial effects and immunomodulatory benefits. Its combination with conventional antibiotics may represent a promising strategy to enhance treatment outcomes and mitigate the growing challenge of antibiotic resistance.

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