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COMPARATIVE EVALUATION OF INFLUENCE OF DIFFERENT CONCENTRATIONS OF CHLORHEXIDINE MOUTHRINSES AGAINST PORPHYROMONAS GINGIVALIS AND FUSOBACTERIUM NUCLEATUM: AN IN-VITRO STUDY

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ABSTRACT

Background: Periodontal pathogens such as Porphyromonas gingivalis and Fusobacterium nucleatum are most dominant in the aetiology of chronic periodontitis and peri-implantitis. This in-vitro study determined the time-dependent bactericidal efficacy of two most used chlorhexidine products (0.2% and 0.12%) against these pathogens under anaerobic conditions. **Methods:** Standardized suspensions of F. nucleatum and P. gingivalis were treated with 0.2% and 0.12% chlorhexidine mouthrinses and 0.9% saline, used as control. All experimental conditions were tested in triplicates, and microbial inocula were grown on blood agar and incubated at 37°C in a CO_2 anaerobic chamber. Microbial quantification of colony-forming units (CFUs) was performed at 0, 1, 2, 5, 10, and 15 minutes. One-way ANOVA with post-hoc Tukey tests were utilized for data analysis, and significance was established at $p < 0.05$. **Results:** The 0.2% chlorhexidine mouthrinse showed an immediate and complete reduction in F. nucleatum at 1 minute (CFU reduction from 131.3 ± 3.51 to 0.0 ± 0.0; $p < 0.001$), while the 0.12% solution showed complete kill of bacteria by 5 minutes, with a more gradual early reduction (113.3 ± 9.07 to 88.6 ± 3.05 at 2 minutes; $p < 0.001$). Saline had no significant reductions at all time points. With P. gingivalis, both concentrations of chlorhexidine showed total bactericidal activity at 5 minutes. Interestingly, the 0.12% preparation showed more reduction between 1 and 2 minutes (214.33 ± 10.96 to 62.66 ± 5.03), while the 0.2% rinse showed more of a gradual reduction (284.33 ± 7.63 to 199.3 ± 6.02 at 2 minutes). **Conclusion:** Both concentrations of chlorhexidine exhibited excellent antimicrobial activity against F. nucleatum and P. gingivalis with complete killing in 5 minutes. The 0.2% solution showed faster bactericidal activity, especially against F. nucleatum, which worked better with short contact times.

KEYWORDS: Chlorhexidine, Porphyromonas gingivalis, Fusobacterium nucleatum, antimicrobial efficacy, mouthrinse, periodontal pathogens, in-vitro study, colony-forming units, bacterial reduction, time-dependent activity.

INTRODUCTION

Periodontal diseases are chronic inflammatory conditions of tooth support tissues, most commonly caused by the development and establishment of subgingival biofilms with certain periodontopathogenic microorganisms. Among the primary bacterial species that cause initiation and progression of periodontitis are Porphyromonas gingivalis and Fusobacterium nucleatum, both anaerobic gram-negative rods with characterized virulence factors.[1,2] P. gingivalis is identified as a keystone

pathogen with the ability to impair host immune function and promote microbial dysbiosis, while F. nucleatum is a bridge species that promotes co-aggregation and structural support of subgingival biofilms.[3,4]

The primary treatment of preference in the prevention and management of periodontal diseases is mechanical plaque control. Patient non-compliance, anatomical constraints, and inaccessibility of certain oral sites frequently require the application of chemical adjuncts for enhanced plaque control and microbial load reduction.[5] Among all the chemical agents, chlorhexidine (CHX), a bisbiguanide antiseptic, has been proven to be the gold standard in oral antimicrobial therapy due to its wide-spectrum bactericidal activity, substantivity, and tissue compatibility.[6] Chlorhexidine acts by interfering with bacterial cell membrane, increasing cellular permeability, and precipitating intracellular material, finally leading to the death of the microorganisms.[7] Further, its ability to bind to oral tissues and release its active ingredients slowly over a period of time ensures long-lasting antimicrobial effects, making it suitable for short-term intensive treatment regimens in periodontal therapy.[8]

Chlorhexidine mouthrinses come in different concentrations, the most commonly used being 0.2% and 0.12%, with variations in composition depending on regional regulation and manufacturer requirements. Despite the long-standing conventional wisdom that 0.2% is a stronger solution, several clinical and in vitro studies have proven that 0.12% solutions are as effective antimicrobially with fewer side effects such as tooth staining, taste modification, and mucosal irritation.[9,10] Despite its widespread use, the concentration-dependent antimicrobial effect of chlorhexidine, particularly against leading periodontal pathogens such as P. gingivalis and F. nucleatum, remains an ongoing focus of investigation. Comparison of microbial susceptibility to different CHX concentrations can provide valuable information for maximizing treatment regimens and minimizing side effects without loss of efficacy.[11]

Therefore, in this study, an in vitro comparison was made of the antimicrobial action of two strengths of the commercially used chlorhexidine mouthwash—0.2% and 0.12%—against standardised strains of Porphyromonas gingivalis and Fusobacterium nucleatum. The research used 0.9% saline as a negative control with the aim of testing the time-dependent bactericidal action of each of the preparations under controlled laboratory conditions.

MATERIALS AND METHODS

Study Design

The current research was designed as an in-vitro comparative experimental study to evaluate and compare the antimicrobial efficacy of two concentrations of chlorhexidine mouthrinses—0.2% and 0.12%—against Porphyromonas gingivalis and Fusobacterium nucleatum. A 0.9% sterile normal saline solution was also used as a negative control. The research was conducted within a microbiology laboratory, under strictly controlled aseptic and anaerobic conditions.

Sample Size and Replication

An official calculation of a sample size was not required since the experiment was in-vitro. Nevertheless, to ensure reliability as well as reproducibility, each of the 3 treatment conditions of each of the bacterial strains was tested in triplicate, and this meant a total of 18 plates for each species of bacterium (3 treatment groups × 6 time intervals × 3 repetitions). This ensured thorough intergroup as well as intragroup analysis across different time points.

Test Materials

The materials used in the study included:

Figure 1: 0.2% chlorhexidine digluconate mouthwash.

- 0.2% chlorhexidine digluconate mouthwash (Figure 1)

Figure 2: 0.12% gluconate chlorhexidine mouthwash.

- 0.12% gluconate chlorhexidine mouthwash (Perioguard) (Figure 2)

Figure 3: 0.9% sterile normal saline solution.

- 0.9% sterile normal saline solution (negative control, Figure 3)

All solutions were stored at room temperature and handled aseptically. Blood agar was employed as the growth medium for microbes and was poured and plated into sterile petri plates.

Microorganisms

The test microorganisms were

- Porphyromonas gingivalis (type strain ATCC)
- Fusobacterium nucleatum (ATCC type strain)

They are both anaerobic gram-negative periodontal pathogens that are most frequently associated with chronic periodontitis and peri-implantitis. The strains were reactivated in suitable anaerobic broth media and incubated at 37°C for 24 hours to provide exponential phase growth before use in experiments.

Preparation of Inoculum and Treatment

For every bacterial strain, an equal amount of microbial suspension of 0.5 McFarland turbidity was prepared and then mixed with each test solution (0.2% CHX, 0.12% CHX, and saline) in separate sterile tubes. Immediately after the mixing, an equal inoculum was inoculated on blood agar plates using sterile cotton swabs.

Incubation Process

Colony-forming units (CFUs) were enumerated at baseline (0 minutes) right after streaking. The agar plates were incubated in a CO_2 anaerobic jar at 37°C, kept constant to replicate the physiological condition of the oral cavity in humans. Plates were taken out successively at 1, 2, 5, 10, and 15 minutes, and colony counts were enumerated on a digital colony counter. Plates were replaced right away in anaerobic conditions to avoid variability according to exposure.

Outcome Measurement

The major endpoint assessed was the decrease in viable bacterial colonies (CFUs) at different times. CFU counts at every time point were compared with baseline within each group and between groups to identify the relative antimicrobial action of each treatment solution against both bacteria.

Quantitative Analysis

Data were tabulated in Microsoft Excel and then exported to SPSS Version 26.0 (IBM Corp., Armonk, NY) for statistical analysis. Descriptive statistics expressed as mean ± standard deviation were calculated. Group comparisons were assessed using one-way ANOVA, with post-hoc Tukey tests. A p-value of less than 0.05 was considered to be statistically significant, and a p-value of less than 0.001 was considered to be highly significant.

RESULTS

Figure 4 shows the antimicrobial effect of 0.2% chlorhexidine digluconate against Porphyromonas gingivalis after various time intervals. There was a dense population of discrete colonies at the initial reading, which was a measure of active growth. After 1 minute's exposure to the antiseptic, there was a marked reduction in colony density with some viable colonies remaining. After 2 minutes' exposure, there was no trace of microbial activity, and after 5 minutes' exposure, the agar surface was devoid of colonies, indicating complete elimination of bacteria. A 10-minute and 15-minute exposure had no trace of regrowth, thus establishing the long-term antimicrobial efficacy of 0.2% chlorhexidine.

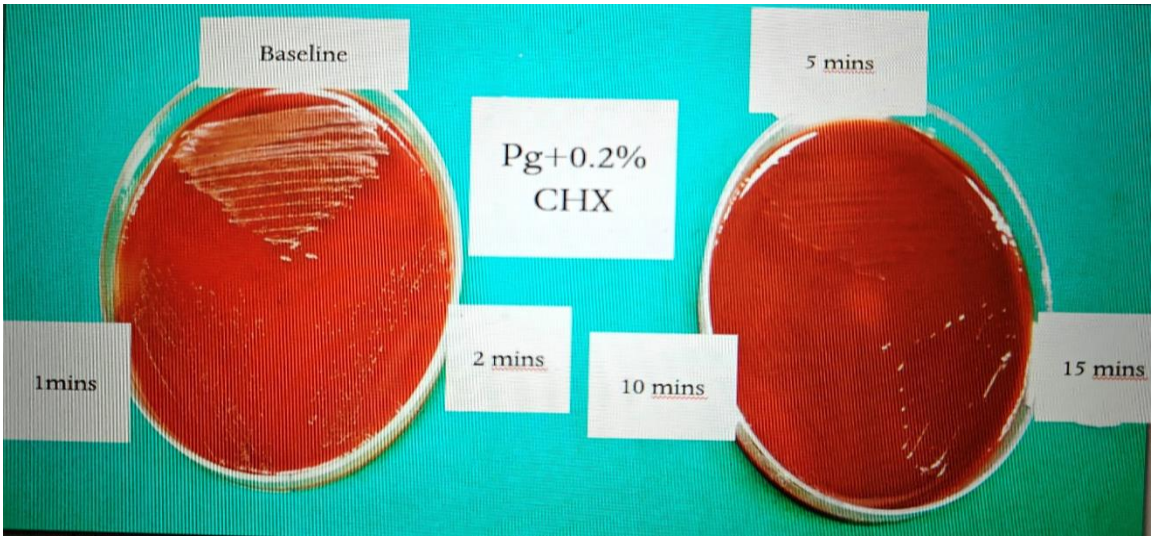

Figure 4: Petri dish containing colonies of P. gingivalis in presence of 0.2% concentration of Chlorhexidine.

Figure 5 illustrates the action of P. gingivalis against a 0.12% chlorhexidine gluconate solution. Initial growth of the bacteria was high and even. At the 1-minute marking, partial inhibition of the colonies with uneven clearing on the agar was observed, whereas at the 2-minute marking, there was a significant loss of colonies, marking the onset of the rapid clearing of the microorganisms. Complete clearing of colonies was observed from the 5-minute marking onwards, which was sustained at the 10 and 15-minute markings, thus proving the effective and sustained bactericidal activity of the 0.12% formulation, although it was comparatively slower in action than its 0.2% counterpart.

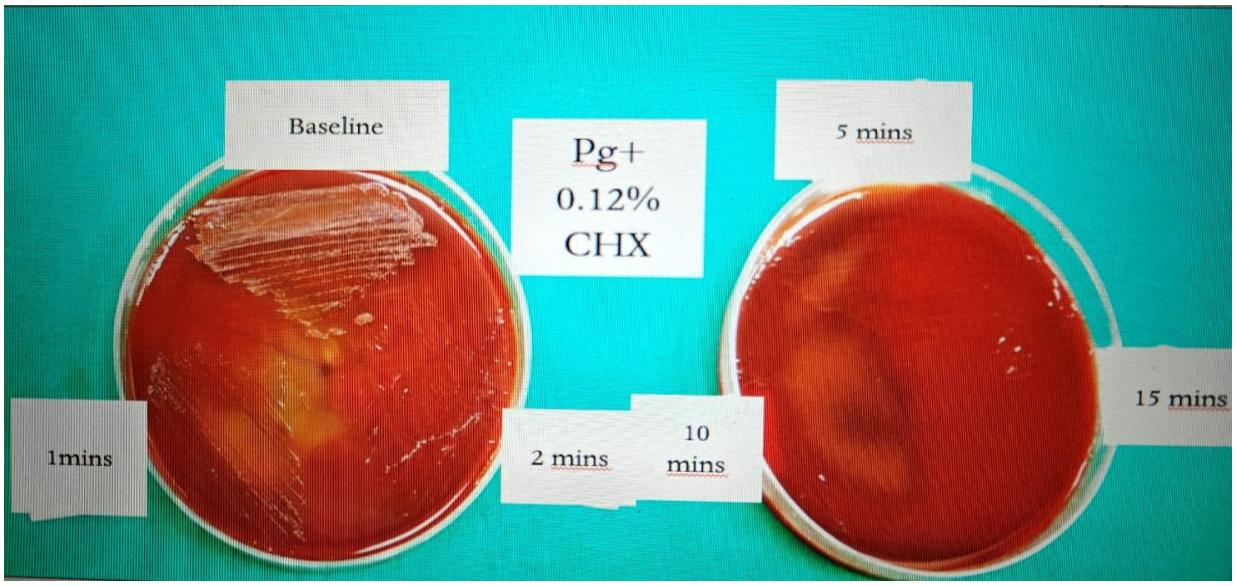

Figure 5: Petri dish containing colonies of P. gingivalis in presence of 0.12% concentration of Chlorhexidine.

Figure 6 shows the growth curve of Porphyromonas gingivalis in normal saline (NS), the negative control of the experiment. There was robust and consistent colony growth initially. This growth curve was consistent across all the time intervals—1, 2, 5, 10, and 15 minutes—without a major decline in the number of bacteria. Streaks of the continuity and density of the colonies indicated that normal saline did not exert bactericidal or bacteriostatic effect against P. gingivalis even with prolonged exposure.

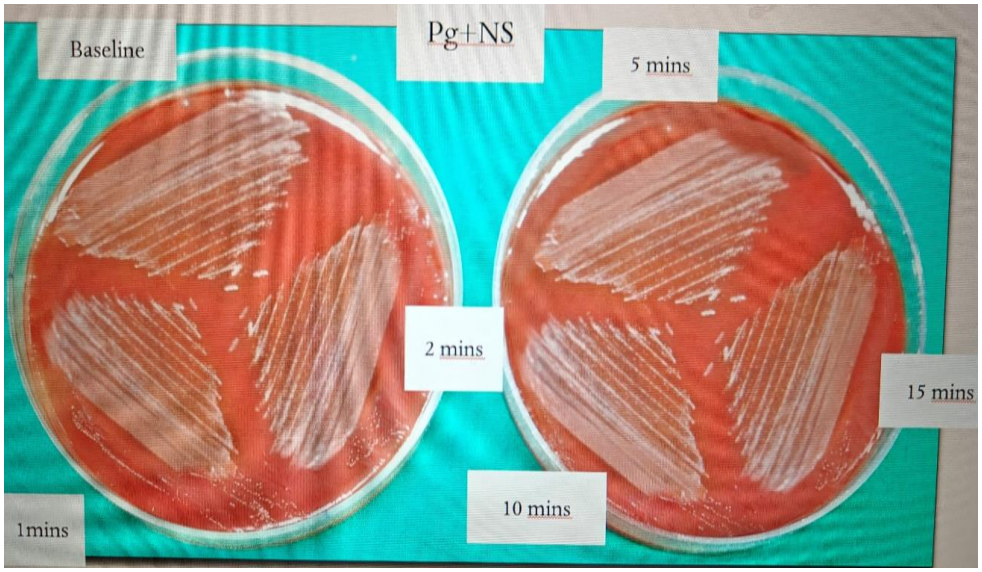

Figure 6: Petri dish containing colonies of *P. gingivalis* in presence of normal saline.

Figure 7 illustrated the action of 0.2% chlorhexidine digluconate against Fusobacterium nucleatum. Baseline showed dense colony formation, which indicated active bacterial growth. At 1 minute of exposure, there was complete colony removal, with the agar surface looking completely clear. This absence of regrowth was seen at all other intervals (2, 5, 10, and 15 minutes), which ensured that 0.2% chlorhexidine had an immediate and prolonged bactericidal action against F. nucleatum.

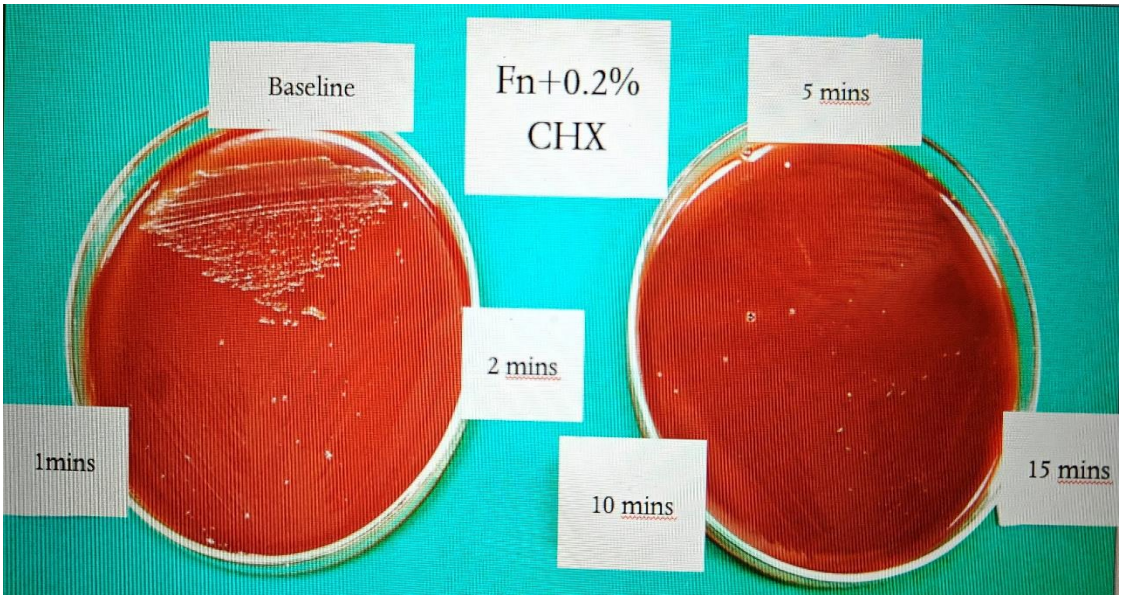

Figure 7: Petri dish containing colonies of *F. nucleatum* in presence of 0.2% concentration of Chlorhexidine.

Figure 8 showed the antimicrobial action of F. nucleatum against 0.12% chlorhexidine gluconate. At baseline, there was substantial bacterial growth. At 1 minute, there were remaining colonies but less densely occupied. There was a noteworthy decrease at 2 minutes, and by 5 minutes, the agar surface was free of growth. No regrowth was seen up to 10 and 15 minutes, which showed that although 0.12% chlorhexidine was not associated with immediate kill within the first minute, it was associated with total bactericidal activity by 5 minutes and continued to do so.

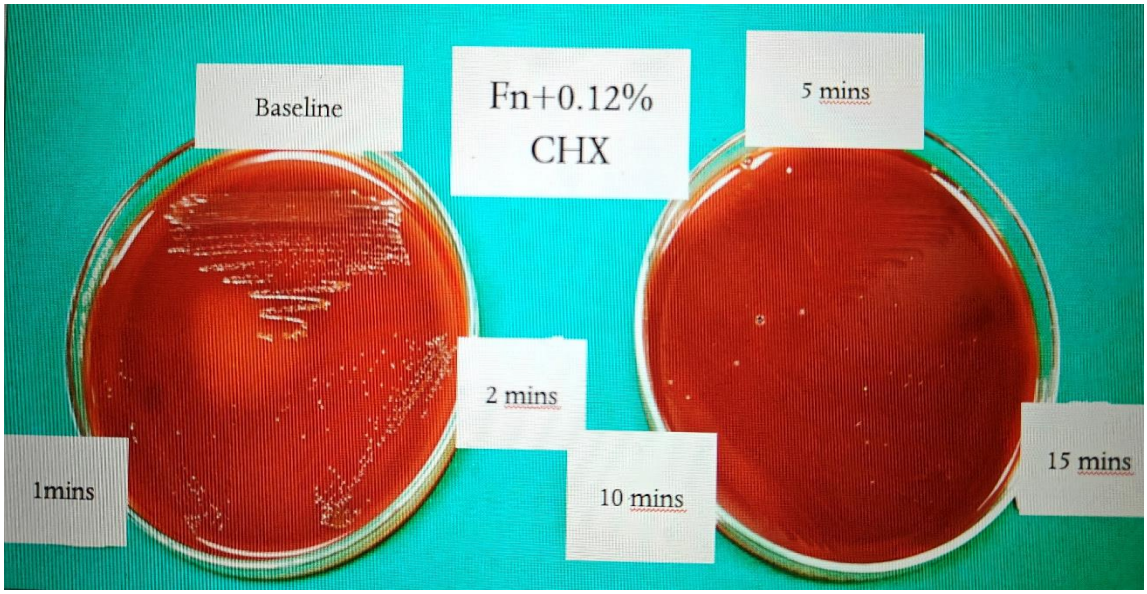

Figure 8: Petri dish containing colonies of *F. nucleatum* in presence of 0.12% concentration of Chlorhexidine.

Figure 9 shows F. nucleatum's reaction to normal saline. In line with the findings reported for P. gingivalis in Figure 6, there was robust colony growth at baseline and continued to be so throughout the period of all time points taken. Thick growing streaks of bacteria were always observed at 1, 2, 5, 10, and 15 minutes, showing that saline did not exert any inhibitory effect on the viability of F. nucleatum.

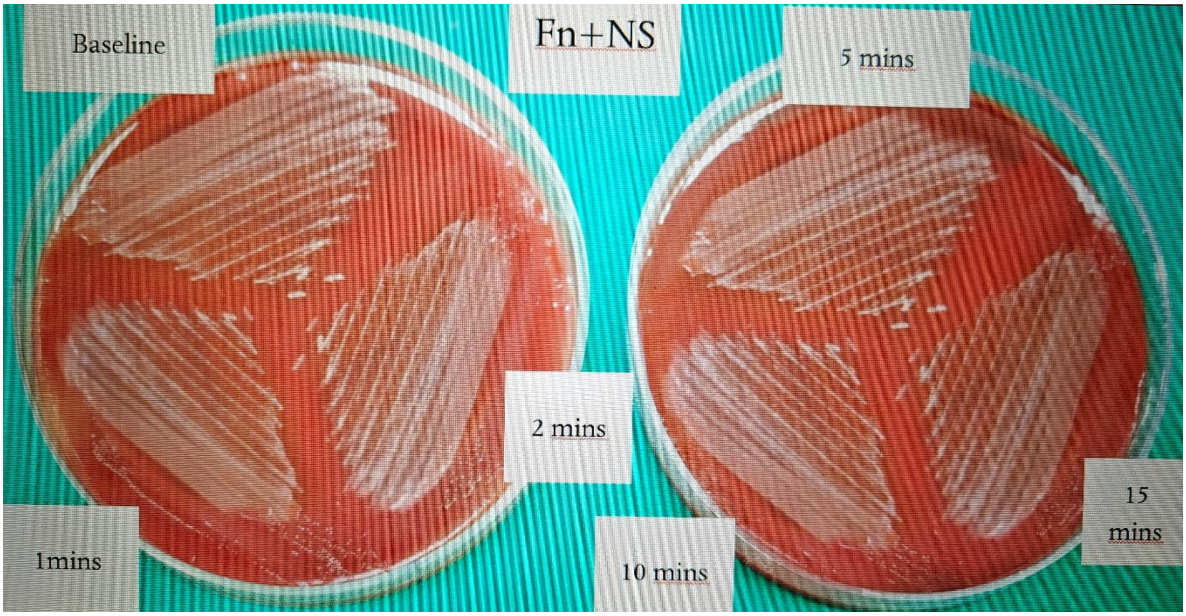

Figure 9: Petri dish containing colonies of *F. nucleatum* in presence of Normal Saline.

For Fusobacterium nucleatum, the 0.2 % chlorhexidine solution (Group A) caused an immediate bactericidal effect: CFUs decreased from 131.3 ± 3.51 at baseline to zero at 1 min and were undetectable thereafter (Table 1). The omnibus test overall was extremely significant ($p < 0.001$), and pair-wise comparisons verified that Group A was superior to both the 0.12 % rinse (Group B) and saline control (Group C) at all post-baseline measurements. Group B did demonstrate a slow but progressive kill—CFUs decreased from 113.3 ± 9.07 at baseline to 108.6 ± 4.04 at 1 min and 88.6 ± 3.05 at 2 min, reaching zero only from 5 min. Saline never demonstrated appreciable bactericidal activity, with counts remaining at ~125 CFU throughout. These data demonstrate a clear concentration-dependent difference in rate of bacterial removal, though both chlorhexidine solutions ultimately caused complete suppression of F. nucleatum within 5 min.

Table 1: Antimicrobial activity of 0.2 % chlorhexidine (Group A), 0.12 % chlorhexidine (Group B) and saline control (Group C) against *Fusobacterium nucleatum*.

Time point	Group A Mean (SD)	Group B Mean (SD)	Group C Mean (SD)	*p* (overall)	A vs B	A vs C	B vs C
Baseline	131.3 (3.51)	113.3 (9.07)	125.2 (7.46)	0.041 *	0.03 *	0.37	0.14
1 min	0.0 (0.0)	108.6 (4.04)	125.2 (7.46)	<0.001 **	<0.001 **	<0.001 **	0.19
2 min	0.0 (0.0)	88.6 (3.05)	125.2 (7.46)	<0.001 **	<0.001 **	<0.001 **	0.031 *
5 min	0.0 (0.0)	0.0 (0.0)	125.2 (7.46)	<0.001 **	<0.001 **	<0.001 **	<0.001 **
10 min	0.0 (0.0)	0.0 (0.0)	125.2 (7.46)	<0.001 **	<0.001 **	<0.001 **	<0.001 **
15 min	0.0 (0.0)	0.0 (0.0)	125.2 (7.46)	<0.001 **	<0.001 **	<0.001 **	<0.001 **

[*NS = not significant ($p > 0.05$); * = significant ($p < 0.05$); ** = highly significant ($p < 0.001$)]

For Porphyromonas gingivalis, baseline counts were greater in all groups, but trends were otherwise consistent with F. nucleatum (Table 2). The 0.2 % rinse lowered CFUs from 284.33 ± 7.63 to 203.0 ± 6.55 at 1 min and 199.3 ± 6.02 at 2 min, to zero by 5 min. The 0.12 % rinse produced an earlier steeper decline—from 214.33 ± 10.96 to 211.0 ± 7.0 at 1 min and a sudden decline to 62.66 ± 5.03 at 2 min—before also reaching eradication at 5 min. Saline again didn't suppress bacterial viability, with counts still ≥254 CFU. Overall and pairwise p-values were significant (< 0.001) at all time points after baseline, establishing the greater antimicrobial activity of chlorhexidine, especially at the higher concentration, in the first 2 min of contact.

Table 2: Antimicrobial activity of 0.2 % chlorhexidine (Group A), 0.12 % chlorhexidine (Group B) and saline control (Group C) against *Porphyromonas gingivalis*.

Time point	Group A Mean (SD)	Group B Mean (SD)	Group C Mean (SD)	*p* (overall)	A vs B	A vs C	B vs C
Baseline	284.33 (7.63)	214.33 (10.96)	254.3 (12.87)	0.021 *	0.01 *	0.37	0.14
1 min	203.0 (6.55)	211.0 (7.0)	254.3 (12.87)	0.421	0.913	0.031 *	0.003 *
2 min	199.3 (6.02)	62.66 (5.03)	254.3 (12.87)	<0.001 **	0.004 *	0.098	<0.001 **
5 min	0.0 (0.0)	0.0 (0.0)	254.3 (12.87)	<0.001 **	<0.001 **	<0.001 **	<0.001 **
10 min	0.0 (0.0)	0.0 (0.0)	254.3 (12.87)	<0.001 **	<0.001 **	<0.001 **	<0.001 **
15 min	0.0 (0.0)	0.0 (0.0)	254.3 (12.87)	<0.001 **	<0.001 **	<0.001 **	<0.001 **

[*NS = not significant ($p > 0.05$); * = significant ($p < 0.05$); ** = highly significant ($p < 0.001$)]

DISCUSSION

The role of antimicrobial mouthrinses in the treatment of periodontal disease as an adjunct remains an important part of current preventive and therapeutic regimens, especially against the background of polymicrobial composition of subgingival biofilm and its central role in the etiology of disease. Chlorhexidine, with its well-established broad-spectrum action and substantive properties, continues to be one of the most widely used antiseptics in periodontal therapy. There has, however, been increasing awareness that differences in chlorhexidine formulation—ranging from concentration, pH, and excipient composition to vehicle properties—can lead to wide differences in antimicrobial activity under in-vitro as well as clinical conditions.[12] Such formulation-dependent differences require further investigations, especially in assessing bactericidal kinetics against a variety of pathogens of relevance to periodontal health.

The in-vitro experiment described herein provided quantitative and qualitative information on the time-dependent antimicrobial action of chlorhexidine mouthrinses at two typical concentrations—0.2% and 0.12%—against Porphyromonas gingivalis and Fusobacterium nucleatum, key pathogens in periodontal disease pathology. The results indicated that while both concentrations ultimately resulted in total bacterial elimination within an exposure time of 5 minutes, the 0.2% solution exhibited a quicker bactericidal effect, notably within the first minute for F. nucleatum. Conversely, the 0.12% concentration had a time-lagged effect; however, it achieved an equivalent outcome against P. gingivalis at the 2-minute time interval. These findings highlight that the antimicrobial action of

chlorhexidine is concentration- and bacterium-dependent, and that lower concentration can be clinically effective if sufficient exposure time is permitted.

This study supplements existing information on chlorhexidine pharmacodynamics and underscored the potential to personalize antimicrobial treatment protocols based on bacterial species susceptibility and necessity for immediate action. Future clinical research could explore the relationship between these in-vitro kinetic observations and their actual clinical effectiveness intraorally, e.g., in plaque reduction and gingival inflammation improvement. These findings could also be utilized to optimize rinse protocols for patients with different risk profiles, notably those that require short antimicrobial exposure, e.g., preprocedural rinses or postoperative therapy.

The microbial community in infected periodontal pockets and root canals is usually composed of anaerobic bacteria such as F. nucleatum and P. gingivalis and facultative or obligate pathogens depending on the clinical situation and host environment.[13] This diversity complicates standardization of antiseptic protocols. In addition, while new plant and polyphenolic compounds have shown high antimicrobial activity against oral pathogens in vitro[14], their potential in practical applications in clinical environments is hampered by complications of cytotoxicity, stability, and biofilm penetration.[15] Conventional agents such as chlorhexidine, therefore, remain a yardstick for comparative evaluation and development of new formulations.

The bactericidal action of chlorhexidine is marked by its disruption of the membrane, precipitation of cytoplasmic ingredients, and release of intracellular components. These actions have been demonstrated to be dose-related and varying based on the microbial membrane composition.[16] In addition, its synergistic binding to hydroxyapatite and to soft tissues also promotes its substantivity, rendering it ideal for long-term antimicrobial activity, especially in cases where mechanical debridement is unsuccessful.[17] However, increased interest in biofilm-focused testing, such as in complex interkingdom models, suggests that classical planktonic tests are insufficient to establish the clinical effectiveness of antiseptics.[18] The biofilm matrix protective action, coupled with the synergistic action of the different species, leads to an elevated resistance to antimicrobial action, thus the need to ascertain the efficacy of mouth rinses in biofilm conditions.

Naturally occurring agents such as plant extracts, berry juices, and flavonoid preparations have been investigated as alternatives or adjuncts to chlorhexidine because of their antimicrobial and antioxidant properties.[19] Nevertheless, these agents, though they show promising results, do not possess substantivity and spectrum to deliver consistent periodontal benefit, particularly in high-risk patients. In addition, adjunctive antiseptic irrigation, especially in deep pockets, has been linked to modest microbial reduction and cytokine modulation gains but is still inferior to mechanical disruption in most clinical situations.[20]

The host immune response, i.e., the plasticity of the Treg-Th17 balance and dendritic cell signaling activity, is at the core of periodontal inflammation and can be indirectly modulated by the decrease of microbial burden using antiseptics.[21] Even in the absence of any direct host immunity effect by antiseptics, these may induce a more desirable inflammatory profile through the relief of bacterial burden. For this purpose, antimicrobial agents that selectively suppress pathogenic microorganisms without influencing commensal flora balance have been in the spotlight. Recent studies on naturopathic mouthwashes and biofilm modulators reveal that selective inhibition can induce oral microbial homeostasis with the restoration of dysbiosis.[22]

Adjuvant therapy with platelet-rich fibrin (PRF) has been shown to display antimicrobial activity by mechanisms involving leukocytes, implying that host-based biomaterials could be employed to augment the activity of chemical antiseptics in the removal of microbial load.[23] Even the combination of physical disruption technologies (e.g., laser therapy) with chemicals has been reported to enhance microbial removal, revealing synergistic uses of chlorhexidine and other modalities.[24]

Periodontal disinfection regimens, particularly those in advanced cases, would be assisted by employing multi-modal regimens that involve chlorhexidine mouth rinses as part of an extended treatment regimen incorporating scaling, host-modifying agents, and microbiota-directed strategies. Evidence shows that full-mouth disinfection regimens incorporating chlorhexidine are effective in reducing biochemical markers of inflammation, such as calprotectin and osteocalcin, indicating systemic and local anti-inflammatory effects.[25]

But the possibility of oral pathogens developing resistance to multiple antiseptic regimens remains a problem. Development of resistance to chlorhexidine and cross-resistance with other antimicrobials, though infrequent, has been reported after extended in-vitro exposure.[26] The findings are in favor of judicious use of antiseptics, a switch between various preparations where possible, and an avoidance of unnecessary extended use in low-risk subjects. Phytotherapeutic extracts from Sphaeranthus indicus were also studied for their antimicrobial activities against periodontopathogenic agents and have been proposed as a source from which to discover future antimicrobial agents.[27] Although these molecules have not yet been used to replace chlorhexidine in the clinical setting, they provide valuable ideas on the future directions for the creation of highly active and low-toxicity mouthrinses.

Limitations

The study's limitations arise from its in vitro nature, which did not account for the salivary dilution, protein binding, tissue retention, and host immune system interactions that influence antimicrobial action in the body. The exposure environment was controlled and static and thus did not reproduce the dynamic nature of oral cavity complexities. The study was also limited to two bacteria strains and did not account for the influence of more realistic polymicrobial interactions that would otherwise be present in subgingival biofilms.

CONCLUSION

Results of the present investigation indicate that a 0.2% chlorhexidine digluconate mouthwash was more active than a 0.12% chlorhexidine gluconate product against Porphyromonas gingivalis and Fusobacterium nucleatum in the first minute of exposure. When exposure time was prolonged to two minutes, however, the 0.12% chlorhexidine gluconate resulted in significantly higher reduction of P. gingivalis colony counts compared to the 0.2% solution. These results indicate that not only concentration, but also exposure time and the nature of bacteria, affect the antimicrobial activity of chlorhexidine mouthrinses. Therefore, differences in chlorhexidine concentrations do not differentially affect periodontal pathogens, and the need to modify antiseptic therapy according to clinical conditions is emphasized.

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