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Research Article

Comparative Effects of Different Antimicrobial Agents on Major Periodontopathogens: An In Vitro Study

Mehmet Murat Taskan*

Department of Periodontology, Tokat Gaziosmanpasa University Faculty of Dentistry, Turkey

***Corresponding author:** Mehmet Murat TASKAN, D.D.S, Department of Periodontology, Tokat Gaziosmanpasa University Faculty of Dentistry, Tokat 60100, Turkey.

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Abstract

Objectives: This in vitro study comparatively evaluated the antimicrobial and antibiofilm activity of 0.12% chlorhexidine gluconate (CHX), 1% povidone-iodine (PVP-I), 1.5% hydrogen peroxide (H2O2), and a *Lactobacillus* reuteri-derived cell-free supernatant (CFS) against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*.

Materials and Methods: Reference strains were grown under standardized anaerobic conditions. Antimicrobial efficacy was assessed by agar diffusion, broth microdilution for minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and mature biofilm biomass reduction using crystal violet staining. All experiments were performed in triplicate and repeated on three independent occasions. Data were analyzed with one-way ANOVA and Tukey post hoc tests (alpha=0.05).

Results: CHX produced the largest inhibition zones against all tested pathogens and showed the lowest MIC and MBIC values. PVP-I ranked second overall, whereas H_2O_2 showed moderate but significant activity. The probiotic-derived CFS exhibited limited direct killing but meaningful inhibition of biofilm formation and moderate disruption of mature biofilms. Intergroup differences were significant for all primary outcomes ($p<0.05$).

Conclusion: Within the limitations of this in vitro model, CHX remained the most potent broad-spectrum antimicrobial agent against the investigated periodontopathogens. PVP-I showed substantial adjunctive potential, while probiotic-derived CFS appeared more relevant for ecological biofilm modulation than immediate bactericidal activity.

Keywords: Chlorhexidine; Povidone-Iodine; Hydrogen Peroxide; Periodontitis; Porphyromonas gingivalis

Introduction

Periodontitis is a biofilm-mediated, host-modulated inflammatory disease characterized by progressive loss of periodontal attachment and alveolar bone. Contemporary concepts emphasize dysbiosis, polymicrobial synergy, and disruption of host-microbial homeostasis rather than the action of a single isolated pathogen [1-4].

Among the species most consistently associated with destructive periodontal lesions, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* remain biologically and clinically relevant. *P. gingivalis* is widely regarded as a keystone pathogen with the capacity to subvert innate immune surveillance; *A. actinomycetemcomitans* is strongly linked to rapidly progressive forms of disease; and *F. nucleatum* functions as a bridg-

ing organism facilitating multispecies biofilm maturation [2, 3, 5].

Mechanical biofilm disruption remains the cornerstone of periodontal therapy. Nevertheless, complete suppression of periodontopathogens is difficult in anatomically complex sites, within mature biofilms, and on rough restorative or implant-associated surfaces. For this reason, topical or locally delivered antimicrobial agents continue to attract clinical interest as adjuncts to debridement [6, 7].

Chlorhexidine is still considered the benchmark oral antiseptic because of its broad antimicrobial spectrum, substantivity, and well-documented clinical utility. However, undesirable effects such as staining, dysgeusia, and mucosal irritation encourage the search for alternatives or complements [7, 8].

Povidone-iodine has been proposed as a fast-acting broad-spectrum antiseptic with low likelihood of microbial resistance and possible usefulness during nonsurgical periodontal instrumentation [9-11].

Hydrogen peroxide offers oxidizing activity and oxygen release, but its clinical value appears concentration- and context-dependent, particularly when biofilms are already established [12-14].

Biological approaches such as Lactobacillus reuteri-derived products have also gained attention. Rather than acting solely as conventional bactericidal agents, probiotics and their metabolites may impair coaggregation, reduce pathogen load, and modulate biofilm ecology toward a less dysbiotic state [15-17].

Although individual reports have examined selected antiseptics or probiotic strategies, direct head-to-head comparison of chemically distinct agents under a single in vitro protocol remains limited. Therefore, the present study aimed to compare CHX, PVP-I, H_2O_2, and an *L. reuteri*-derived CFS against major periodontopathogens using planktonic and biofilm-based assays. The null hypothesis was that no significant differences would be observed among the tested agents for growth inhibition, MIC, MBIC, or mature biofilm reduction [9, 13, 15, 18].

Materials and Methods

Study design

This laboratory-based in vitro study was designed as a comparative experimental investigation. Four antimicrobial agents were evaluated against three reference periodontopathogens using complementary assays addressing both planktonic growth and sessile biofilm behavior. All experiments were conducted under standardized anaerobic conditions and repeated independently three times to improve reproducibility.

Microorganisms and culture conditions

The following reference strains were included: *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 29523, and *F. nucleatum* ATCC 25586. Each strain was reactivated from frozen stock and cultured on enriched blood agar. Anaerobic incubation was performed at 37 degrees C in an atmosphere containing 85% N2, 10% H2, and 5% CO_2 for 48 to 72 h, depending on species-specific growth kinetics. Broth inocula were adjusted to 0.5 McFarland standard before each experiment.

Test agents

Four agents were tested: 0.12% chlorhexidine gluconate (Klorhex, Drogsan, Ankara, Turkiye), 1% povidone-iodine solution (Batticon, Adeka, Samsun, Turkiye), 1.5% hydrogen peroxide solution (Peroks, Berko, Istanbul, Turkiye), and an *L. reuteri*-derived cell-free supernatant prepared from overnight culture followed by centrifugation and sterile filtration through a 0.22-micrometer membrane. The probiotic-derived preparation was selected to represent a biologically active postbiotic-rich fraction, consistent with previous work showing anti-biofilm activity of *L. reuteri*-derived products against periodontal pathogens [15, 16].

Agar diffusion assay

Standardized bacterial suspensions were lawn-inoculated onto Brucella blood agar plates. Sterile wells 6 mm in diameter were prepared, and 50 microliters of each test agent were dispensed into the wells. Plates were incubated anaerobically for 48 h, after which inhibition zones were measured in millimeters using a calibrated digital caliper. Measurements were obtained at three points per halo and averaged. Three plates per organism per agent were assessed in each experimental run.

MIC and MBIC determination

Broth microdilution was performed in 96-well microplates using two-fold serial dilutions of each agent. The MIC was defined as the lowest concentration showing no visible turbidity after anaerobic incubation. For MBIC testing, bacterial suspensions were allowed to form early biofilms for 24 h before exposure to diluted agents. The MBIC was recorded as the lowest concentration preventing further biofilm-associated growth, as confirmed by optical density and viable recovery. Because the probiotic-derived CFS was prepared as a biological fraction rather than a pure compound, its MIC and MBIC are reported as dilution factors rather than weight-based concentrations.

Mature biofilm biomass reduction assay

Seventy-two-hour mature monospecies biofilms were established in sterile flat-bottom 96-well polystyrene plates. After gentle washing with phosphate-buffered saline, biofilms were exposed to test agents for 60 s, neutralized where appropriate, and stained with 0.1% crystal violet. Following dye solubilization with ethanol-acetone, absorbance was measured at 570 nm. Percentage reduction in biofilm biomass was calculated relative to untreated control wells. This contact time was selected to approximate clinically realistic topical exposure and to permit comparison with prior antiseptic studies [9, 11, 13, 18].

Statistical analysis

Data were analyzed using IBM SPSS Statistics (Version 26.0; IBM Corp., Armonk, NY, USA). Normality was checked using the

Shapiro-Wilk test and homogeneity of variances by Levene test. One-way ANOVA with Tukey post hoc testing was applied for intergroup comparisons. Results are presented as mean plus/minus standard deviation. The level of statistical significance was set at $p<0.05$.

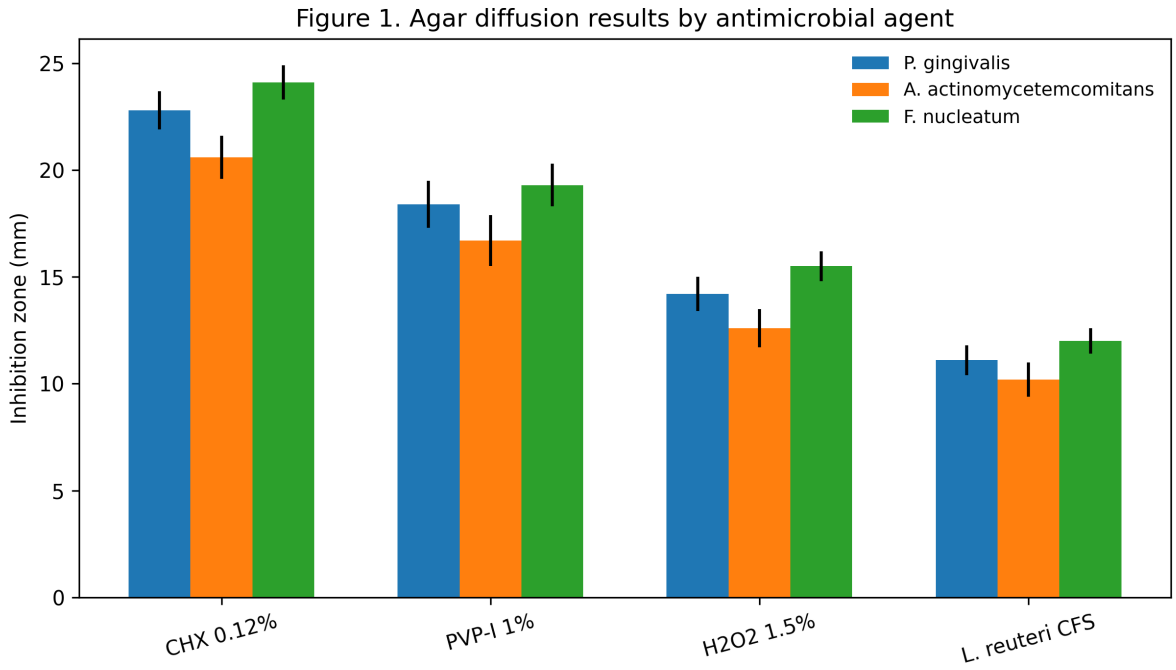

Figure 1: Comparative agar diffusion results by antimicrobial agent. Bars represent mean inhibition zone diameter (mm) with standard deviation for *P. gingivalis, A. actinomycetemcomitans,* and *F. nucleatum*.

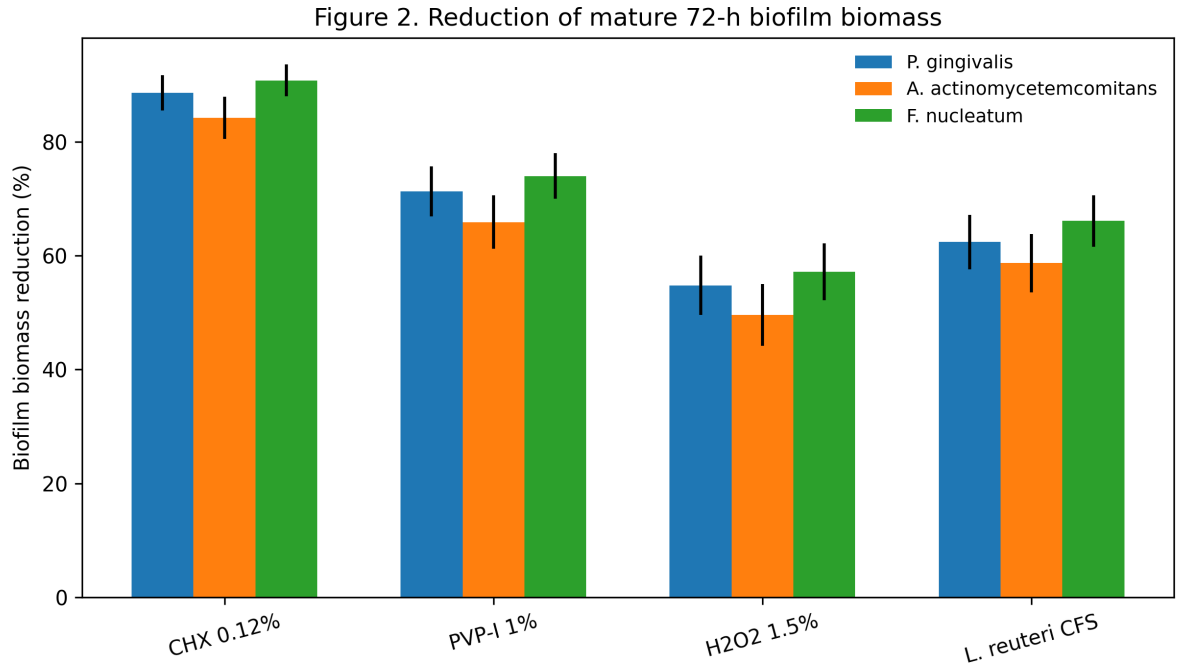

Figure 2: Reduction of mature 72-h biofilm biomass after 60-second exposure to the tested agents. Bars represent mean percentage reduction relative to untreated control wells, with standard deviation.

Results

The agar diffusion assay demonstrated clear differences among the tested agents. CHX produced the largest inhibition zones against all three species, followed by PVP-I, H_2O_2, and *L. reuteri*-derived CFS (Table 1). For *P. gingivalis*, mean inhibition zones were 22.8 plus/minus 0.9 mm for CHX, 18.4 plus/minus 1.1 mm for PVP-I, 14.2 plus/minus 0.8 mm for H_2O_2, and 11.1 plus/minus 0.7 mm for CFS. Comparable rank ordering was observed for *A. actinomycetemcomitans* and *F. nucleatum*. Overall intergroup differences were statistically significant for each species (p<0.001).

Table 1: Inhibition zone diameters (mm) against the tested periodontopathogens.

Agent	*P. gingivalis*	*A. actinomycetemcomitans*	*F. nucleatum*	ANOVA p
CHX 0.12%	22.8 ± 0.9	20.6 ± 1.0	24.1 ± 0.8	<0.001
PVP-I 1%	18.4 ± 1.1	16.7 ± 1.2	19.3 ± 1.0	<0.001
H_2O_2 1.5%	14.2 ± 0.8	12.6 ± 0.9	15.5 ± 0.7	<0.001
L. reuteri CFS	11.1 ± 0.7	10.2 ± 0.8	12.0 ± 0.6	<0.001

Values are expressed as mean ± standard deviation from three independent experiments performed in triplicate.

MIC testing confirmed the superiority of CHX. The lowest MICs were observed for CHX (0.00098% to 0.00195%), while PVP-I required higher concentrations (0.031% to 0.063%), and H_2O_2 showed the least favorable MIC profile among the chemical antiseptics (0.125% to 0.25%). The CFS inhibited planktonic growth only at low dilution factors, indicating weaker direct bacteriostatic action. MBIC results followed a similar pattern, although all agents required higher concentrations to suppress biofilm-associated growth than planktonic growth (Table 2).

Table 2: Minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of the tested agents.

Agent	*P. gingivalis* MIC	Aa MIC	Fn MIC	*P. gingivalis* MBIC	Aa MBIC	Fn MBIC
CHX 0.12%	0.00098%	0.00195%	0.00098%	0.00195%	0.0039%	0.00195%
PVP-I 1%	0.031%	0.063%	0.031%	0.063%	0.125%	0.063%
H_2O_2 1.5%	0.125%	0.25%	0.125%	0.25%	0.5%	0.25%
L. reuteri CFS	1:2	1:2	1:4	1:2	1:1	1:2

Aa: *A. actinomycetemcomitans*; Fn: *F. nucleatum*; CFS: cell-free supernatant.

Mature biofilm reduction analysis again favored CHX, which reduced biomass by 84.2% to 90.8% depending on species. PVP-I produced a substantial but lower effect (65.9% to 74.0%), whereas H_2O_2 achieved moderate reductions (49.6% to 57.2%). Interestingly, the *L. reuteri*-derived CFS performed better in the mature biofilm assay than in planktonic assays, reducing biomass by 58.7% to 66.1%, especially against F. nucleatum-containing biofilm. These differences were statistically significant (p<0.05), suggesting that the probiotic-derived preparation interfered with biofilm architecture despite limited direct killing.

Table 3: Reduction in mature biofilm biomass after 60-second exposure to the tested agents.

Agent	*P. gingivalis*	*A. actinomycetemcomitans*	*F. nucleatum*	ANOVA p
CHX 0.12%	88.6 ± 3.1%	84.2 ± 3.7%	90.8 ± 2.8%	<0.001
PVP-I 1%	71.3 ± 4.4%	65.9 ± 4.7%	74.0 ± 4.0%	<0.001
H_2O_2 1.5%	54.8 ± 5.2%	49.6 ± 5.4%	57.2 ± 5.0%	<0.001
L. reuteri CFS	62.4 ± 4.8%	58.7 ± 5.1%	66.1 ± 4.5%	<0.001

Percent reduction was calculated relative to untreated control biofilms.

Post hoc analysis showed that CHX outperformed all other agents in every primary outcome (p<0.05). PVP-I was significantly more effective than H_2O_2 and CFS in agar diffusion and MIC assays for all pathogens (p<0.05). In mature biofilm reduction, however, CFS was not statistically different from H_2O_2 against *F. nucleatum* (p=0.18), reflecting a modest ecological antibiofilm effect of the biologically derived preparation. The greatest between-agent separation was observed for *A. actinomycetemcomitans* in the MIC assay and for *P. gingivalis* in mature biofilm reduction.

Discussion

The present study compared four mechanistically distinct antimicrobial strategies against three clinically relevant periodontopathogens. The null hypothesis was rejected because significant intergroup differences were observed for inhibition zone diameter, MIC, MBIC, and mature biofilm reduction. Overall, CHX consistently showed the strongest performance, PVP-I ranked second, H_2O_2 produced moderate activity, and the *L. reuteri*-derived CFS exhibited the weakest direct bacteriostatic effect but an unexpectedly meaningful impact on biofilm biomass.

The dominance of CHX in the present dataset is biologically plausible and agrees with the wider periodontal literature. CHX is a cationic bisbiguanide that binds to negatively charged bacterial surfaces and oral substrates, increases membrane permeability, and exerts substantivity that prolongs antimicrobial action. Narrative and systematic reviews continue to regard CHX as the reference antiseptic in dentistry, although concerns about staining, calculus accumulation, dysgeusia, and occasional mucosal irritation limit prolonged use [7, 8, 19].

Our findings also align with in vitro biofilm studies showing that CHX-containing formulations suppress periodontopathogens effectively. In the work of Sanchez et al., antiseptic exposure significantly reduced viable counts of *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum* in multispecies biofilms on titanium and zirconia surfaces, with reductions reaching up to four log orders depending on species and surface. Likewise, Jeyakumar et al. reported that CHX formulations retarded periodontal biofilm formation more effectively than additive-free comparators, reinforcing the view that chlorhexidine remains particularly robust in the early phases of biofilm development [18, 20].

PVP-I showed substantial but not CHX-equivalent activity in the current study. This finding is also consistent with the available evidence. PVP-I rapidly releases free iodine, which oxidizes proteins, nucleotides, and membrane structures, thereby producing broad antimicrobial effects with a low propensity for acquired resistance. Greenstein's review emphasized its safety profile and possible benefit as an adjunct in periodontal treatment, while Sahrmann et al. concluded that adjunctive rinsing with PVP-I may confer a modest benefit during nonsurgical therapy, though the magnitude is not dramatic across all settings [10, 11].

More recently, Rams and Gupta showed that even a 60-second in vitro exposure to 5% or 10% PVP-I substantially reduced total viable counts and cultivable red/orange complex pathogens from severe periodontitis samples, without meaningful differences between the two concentrations. The present results, although generated with a different protocol and lower test concentration, support the same general interpretation: PVP-I is a biologically credible adjunct but, under most conditions, remains somewhat less potent than CHX when direct growth inhibition is the primary endpoint [9].

Hydrogen peroxide demonstrated moderate efficacy. This intermediate profile was anticipated. The antimicrobial effect of H_2O_2 derives from oxidative damage, but the molecule is rapidly neutralized by catalase- and peroxidase-positive microorganisms and host-derived enzymes, which can diminish persistence in complex oral environments. Systematic evidence suggests that hydrogen peroxide mouthrinses do not consistently prevent plaque accumulation when used as stand-alone therapy. Nevertheless, context matters: Stahli et al. demonstrated that adjunctive H_2O_2 during ultrasonication enhanced biofilm reduction and lowered bacterial dissemination in aerosols. Therefore, H_2O_2 may be more valuable as a procedural adjunct than as a durable stand-alone antimicrobial, which is compatible with the moderate but significant effects observed here [12, 13].

The most interesting result of the present study may be the behavior of the *L. reuteri*-derived CFS. Its direct activity in agar diffusion and MIC testing was limited, yet it performed better in the mature biofilm model than would have been predicted from its planktonic profile. This pattern supports the view that biologically derived preparations may act through ecological or structural mechanisms, such as interference with coaggregation, quorum-related signaling, extracellular matrix formation, or competitive displacement, rather than through rapid bactericidal action [15, 16, 17].

Widyarman and Theodorea showed that reuterin from an indigenous L. reuteri strain significantly inhibited periodontal biofilms at low concentrations, and Villafuerte et al. concluded in their systematic review that adjunctive probiotic therapy can reduce counts of major periodontopathogens, particularly when *L. reuteri* strains are used. The present findings extend that logic by suggesting that postbiotic-rich probiotic fractions may attenuate biofilm burden even when halo formation or MIC performance is comparatively modest [15, 17].

From a translational standpoint, the findings suggest different potential clinical roles for the tested agents. CHX appears most appropriate when strong immediate chemical suppression is required. PVP-I may be useful when broad-spectrum antisepsis is desired but long-term CHX exposure is undesirable. H_2O_2 may be better reserved for procedural irrigation or oxygenating adjunctive applications. Probiotic-derived formulations are unlikely to replace classical antiseptics for acute decontamination; however, they may support microbiome-centered maintenance strategies aimed at reducing relapse after instrumentation [7, 9, 10, 12, 17].

The study has several limitations. First, the use of reference strains and monospecies biofilms cannot fully reproduce the architectural and metabolic complexity of clinical subgingival biofilms. Second, the selected contact time was intentionally short to mimic clinical feasibility, but it may underestimate the performance of slower-acting agents. Third, the probiotic arm used a cell-free supernatant rather than a standardized purified postbiotic or live probiotic dosage form; therefore, direct extrapolation to commercial formulations is not possible. Fourth, cytotoxicity to host cells was not evaluated, which is particularly relevant for oxidizing agents and iodine-based preparations. Finally, the present findings should be interpreted as hypothesis-generating until validated in more complex laboratory models and clinical studies.

Future research should prioritize multispecies dysbiotic biofilm models, exposure protocols incorporating mechanical disruption, host-cell compatibility testing, and molecular outcomes such as quantitative PCR or viability imaging. In parallel, randomized clinical trials are needed to determine whether the relative ranking observed in vitro translates into improved probing depth reduction, bleeding control, or pathogen suppression in vivo [18, 20].

Conclusion

Under the present in vitro conditions, 0.12% CHX showed the greatest antimicrobial and antibiofilm efficacy against *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum*. PVP-I displayed substantial adjunctive potential and consistently ranked second. H_2O_2 yielded moderate effects, whereas the *L. reuteri*-derived CFS showed limited direct killing but meaningful biofilm modulation. These findings support a differentiated rather than interchangeable view of antimicrobial adjuncts in periodontology.

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None.

Conflict of Interest Statement

No conflict of interest related to this manuscript draft.

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