ANTIMICROBIAL ACTIVITY OF AN INNOVATIVE CHLORHEXIDINE-FREE CHILDREN'S MOUTHWASH AGAINST BENEFICIAL AND PATHOGENIC ORAL MICROORGANISMS

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ABSTRACT

The aim of the present study was to evaluate the in vitro antimicrobial efficacy of an experimentally developed chlorhexidine-free children's mouthwash against selected beneficial and pathogenic oral microorganisms. The antimicrobial activity of individual components and combined mouthwash formulations was assessed using agar diffusion, direct-contact tests, synchronous cultivation and time-kill assays with mixed microbial suspensions and donor-derived dental plaque. The antimicrobial effect was strain-dependent: Tannerella forsythia and Prevotella intermedia showed the highest resistance, whereas Fusobacterium nucleatum and Streptococcus salivarius were more susceptible to the tested components. The prototype formulation demonstrated substantial antimicrobial activity against all tested microorganisms. In the direct-contact test, Sample 1 achieved a 57.22 % reduction in viable cells after 90 s of exposure in the mixed pathogen population, compared with 67.91 % for Eludril classic® and 33.90 % for the placebo formulation. The overall bacterial death-rate constant of Sample 1 was lower than that of the chlorhexidine-containing reference product but remained markedly higher than that of the placebo. The tested multicomponent chlorhexidine-free formulation exhibited relevant antimicrobial activity against planktonic and biofilm-associated oral microorganisms, supporting its potential as a well-tolerated adjunct for pediatric preventive oral care.

Keywords: mouthwash, cetylpyridinium chloride, chlorhexidine-free formulation, oral biofilm, pediatric dentistry, antimicrobial activity.

INTRODUCTION

Orthodontic treatment in childhood and adolescence has become increasingly common because of its established role in functional correction, aesthetics and psychosocial development. At the same time, fixed orthodontic appliances create retentive areas that facilitate dental plaque accumulation and the development of a structured oral biofilm [1 - 3]. This substantially increases the risk of enamel demineralization, white spot lesions and gingival inflammation [4], [5]. In younger patients, these risks are additionally amplified by still-developing oral hygiene habits, frequent carbohydrate intake and the difficulty of cleaning around brackets and orthodontic elements [6, 7].

Mechanical oral hygiene remains the main method of plaque control, but it is often insufficient in difficult-to-reach sites around orthodontic appliances [8, 9]. This creates the need for adjunctive chemical plaque-control measures capable of reducing microbial load and suppressing inflammation [10 - 12]. Mouthwashes are a convenient and widely accepted means of delivering active compounds to retentive areas [10, 11].

Many commercially available products contain

strong antiseptics that may not be suitable for prolonged use in children because of undesirable side effects [13, 14]. Therefore, there is a need to develop formulations with proven antimicrobial activity and a favorable safety and tolerability profile for pediatric use [15]. The present study was designed to evaluate an innovative mouthwash formulation under laboratory conditions.

The aim of the study was to evaluate the in vitro antimicrobial efficacy of individual active ingredients and an experimentally developed children's mouthwash against beneficial and pathogenic oral microorganisms under conditions approximating the oral environment.

EXPERIMENTAL

Study design

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Plovdiv, Bulgaria, Approval No. P-KNE-17/31.12.2024, Protocol No. 9/28 November 2024.

The experimental work was organized in three consecutive phases. Phase 1 included activation and individual cultivation of the following oral microorganisms: *Tannerella forsythia* DSM 102835, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 25586, *Enterococcus faecalis* ATCC 29212 and *Streptococcus salivarius* ATCC 13419.

Phase 2 involved synchronous mixed cultivation of the tested periodontal pathogens together with representatives of the oral microbiota. Donor dental plaque samples were also cultivated as a model of clinical biofilm.

Phase 3 included testing of individual components and prototype mouthwash formulations. The individual components and complete formulations were evaluated using agar diffusion and direct-contact assays under predefined experimental conditions. Short-exposure antimicrobial activity was assessed at 30 s, 60 s and 90 s.

Tested materials

The study included the following individual components and formulations: chlorhexidine, cetylpyridinium chloride (CPC), Aloe vera, sodium fluoride, inulin, hyaluronic acid, allantoin, glycerin, stevia, poloxamer 407, sodium benzoate, citric acid, experimental children's mouthwash - Sample 1, placebo formulation - Sample 0 and Eludril classic®. Sample 1 contained CPC, Aloe vera, inulin, sodium fluoride and technological excipients, whereas Sample 0 lacked active antiseptic compounds.

Culture media and laboratory materials

The microbiological analysis used BHI medium, Lactobacillus MRS broth, M17 broth, agar powder, defibrinated sheep blood and anaerobic cultivation systems. All Petri dishes, tubes and pipettes were sterile.

Assessment of antimicrobial activity

For the assessment of antimicrobial activity, two standard microbiological methods were applied. In the agar diffusion assay, sterile 6 - mm paper discs were loaded with 6 μL of each tested sample. After incubation for 24 h at 37°C, inhibition zone diameters were measured in mm.

In the direct-contact and synchronous cultivation tests, a mixture was prepared by combining 0.5 cm^3 of the tested component or solution, 0.5 cm^3 of pathogen suspension and 9 cm^3 of the corresponding medium. Control samples were prepared using medium and microbial suspension only. Cultivation was carried out under static conditions at 37°C for 60 - 72 h, with sampling at 0, 12, 24, 36, 48, 60 and 72 h.

To evaluate antimicrobial activity under short-exposure conditions, mixed periodontal pathogen suspensions and donor plaque samples were directly added to the tested mouthwashes. Viable cell concentration was determined at 0, 30, 60 and 90 s.

Kinetic evaluation

The overall bacterial death rate constant, Koverall, was calculated using a first-order kinetic model presented in Eq. (1):

$$dN/d\tau = K_{overall}N; \quad lnN = lnN0 - K_{overall}\tau \qquad (1)$$

where N0 is the initial viable cell concentration, N is the current viable cell concentration, τ is the exposure time and Koverall is the overall rate constant of bacterial death.

Statistical analysis

Statistical analysis was performed using SPSS

version 26 (IBM Corp., Armonk, NY, USA). Data are presented as mean ± standard deviation (SD). Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey HSD post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Antimicrobial activity of individual components

The primary outcomes were inhibition zone diameters, viable bacterial counts expressed as Log CFU, percentage reduction in microbial load and the overall bacterial death-rate constant (Koverall).

The agar diffusion assay showed clear strain-specific susceptibility among the tested oral microorganisms. Chlorhexidine exhibited universal and maximal antimicrobial activity, with inhibition zones of 18 ± 0.00 mm against all tested strains. Cetylpyridinium chloride demonstrated activity against all microorganisms, with inhibition zones ranging from 9 to 12 mm. The lowest susceptibility to CPC was observed for *P. gingivalis*. The technological excipients demonstrated weaker and more selective effects, often limited to inhibition zones of 8 - 10 mm or absence of detectable activity.

The highest resistance to the tested components was observed for *T. forsythia* and *P. intermedia*. In contrast, *F. nucleatum* and *S. salivarius* showed susceptibility to a broader spectrum of components. These results indicate that the antimicrobial activity of the individual ingredients was strongly strain dependent. The numerical results are summarized in Table 1 and visualized in Fig. 1.

Antimicrobial activity of prototype mouthwash formulations

Intergroup comparison showed statistically significant differences in antimicrobial activity among the tested mouthwash formulations ($p < 0.05$). Eludril classic® demonstrated the strongest overall antimicrobial profile, whereas Sample 1 showed substantial activity against all tested microorganisms and reached inhibition zones like or higher than those of the reference product for selected strains. The placebo formulation showed the weakest overall effect.

Sample 1 showed higher antimicrobial activity than the placebo formulation for all tested microorganisms. The observed activity of the placebo was minimal and may be related to the non-specific effects of preservatives and technological excipients rather than to a specific antiseptic mechanism. The numerical results are presented in Table 2 and Fig. 2.

Direct-contact test: mixed pathogen population and donor dental plaque

The antimicrobial effect of the tested formulations was evaluated at four exposure time points: baseline, 30 s, 60 s and 90 s. In the mixed pathogen population,

Table 1. Antimicrobial activity of the main components, mm ± SD.

Substance	*P. intermedia*	*P. gingivalis*	*T. forsythia*	*F. nucleatum*	*S. salivarius*	*E. faecalis*
Chlorhexidine	18 ± 0.00	18 ± 0.00	18 ± 0.00	18 ± 0.00	18 ± 0.00	18 ± 0.00
Glycerin	-	8 ± 0.00	-	-	9 ± 0.71	9 ± 0.00
Inulin	-	8 ± 0.00	-	-	-	9 ± 0.00
Hyaluronic acid	-	8 ± 0.00	-	8 ± 0.00	-	8 ± 0.00
Allantoin	-	-	-	8 ± 0.00	-	-
CPC	11 ± 0.00	9 ± 0.00	11 ± 0.00	10 ± 0.00	10 ± 0.00	12 ± 0.00
Sodium fluoride	8 ± 0.00	-	-	-	9 ± 0.00	-
Stevia	8 ± 0.00	-	-	10 ± 0.71	9 ± 0.00	-
Poloxamer 407	-	9 ± 0.00	8 ± 0.00	9 ± 0.00	9 ± 0.71	-
Sodium benzoate	-	-	-	10 ± 0.71	-	-
Citric acid	-	-	-	8 ± 0.00	19 ± 0.71	-
Aloe vera	-	11 ± 0.00	10 ± 0.00	8 ± 0.00	10 ± 0.00	8 ± 0.00
Control	-	-	-	-	-	-

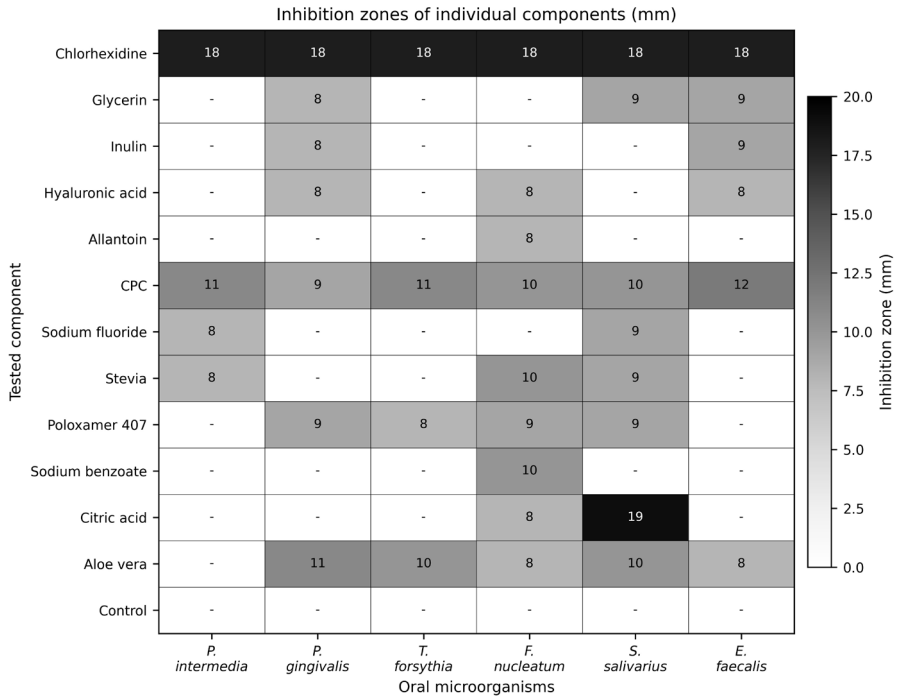


Fig. 1. Heat map of inhibition zones (mm) for the tested individual components against selected oral microorganisms.

Table 2. Antimicrobial activity of mouthwash formulations, mm ± SD.

Sample	*S. salivarius*	*E. faecalis*	*T. forsythia*	*F. nucleatum*	*P. gingivalis*	*P. intermedia*
Eludril classic®	10 ± 0.00	14 ± 0.00	14 ± 0.00	14 ± 0.00	12 ± 0.00	10 ± 0.00
Children's mouthwash - sample 1	11 ± 0.71	11 ± 0.00	14 ± 0.71	12 ± 0.00	11 ± 0.71	14 ± 0.00
Children's mouthwash - sample 0 (placebo)	9 ± 0.71	10 ± 0.00	11 ± 0.00	10 ± 0.00	12 ± 0.00	8 ± 0.00

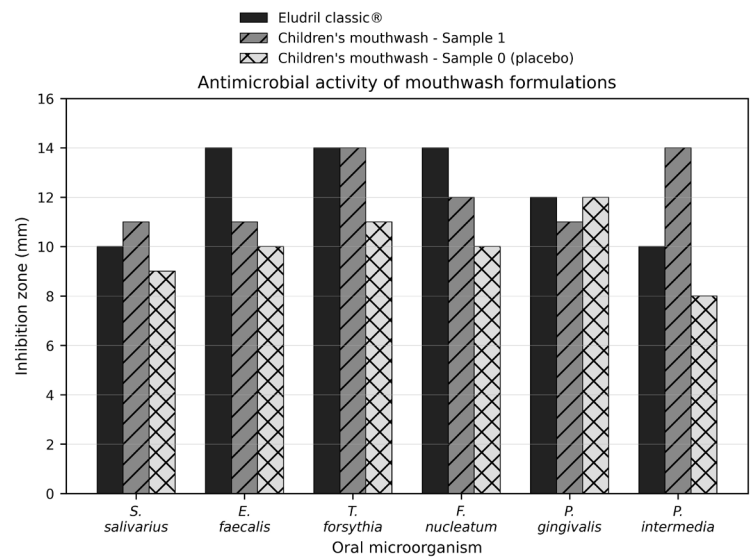


Fig. 2. Comparative antimicrobial activity of prototype and reference mouthwash formulations.

Sample 1 produced a marked reduction in viable cells within the first 30 s, followed by further reduction up to 90 s. The total reduction reached 57.22 %. Eludril classic® showed the highest reduction, reaching 67.91 %, whereas the placebo formulation achieved 33.90 %.

Intragroup comparison across the exposure time points showed a statistically significant time-dependent reduction in viable bacterial counts for the active formulations ($p < 0.05$). The greatest decrease was observed within the first 30 s of exposure, while the reduction between 60 s and 90 s was less pronounced.

Intergroup comparison at the 90 - s endpoint showed that Eludril classic® produced the strongest antimicrobial effect, followed by Sample 1, whereas the placebo showed the lowest reduction in viable cells. Although Sample 1 showed lower overall activity than Eludril classic®, the time-dependent reduction pattern was comparable to that of the reference product.

When donor dental plaque was used instead of the mixed reference strains, a similar time-dependent reduction was observed. However, the reduction was slower and less pronounced than that observed in the free bacterial suspension, indicating higher resistance of microorganisms organized in dental plaque. At 90 s, Sample 1 reduced the viable cell concentration to 4.00 Log CFU, corresponding to a total reduction of 52.33 %, while Eludril classic® achieved a reduction of 67.50 %.

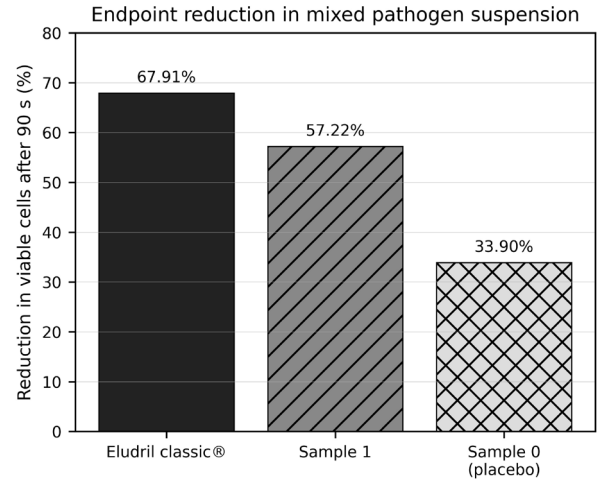


Fig. 3. Endpoint reduction of viable cells after 90 s of direct exposure in mixed pathogen suspension.

Overall bacterial death-rate constant

The overall bacterial death-rate constant confirmed the differences in antimicrobial activity among the tested formulations. Intergroup comparison of Koverall values showed statistically significant differences between Eludril classic®, Sample 1 and the placebo formulation ($p < 0.05$).

Eludril classic® showed the highest Koverall values: 0.151 ± 0.001 s^{-1} for the mixed pathogen population and 0.148 ± 0.001 s^{-1} for donor dental plaque. Sample 1 demonstrated comparatively high values of 0.132 ± 0.001 s^{-1} and 0.123 ± 0.001 s^{-1}, respectively. The placebo formulation showed the lowest Koverall values: 0.075 ± 0.001 s^{-1} for the mixed pathogen population and 0.084 ± 0.001 s^{-1} for donor dental plaque (Table 3, Fig. 4).

These results confirm the following order of antimicrobial efficacy: Eludril classic® > Sample 1 > placebo. Although the bacterial death-rate constant of Sample 1 was lower than that of Eludril classic®, it remained substantially higher than that of the placebo formulation.

Based on the calculated Koverall values, the theoretical exposure time required for Sample 1 to achieve an antimicrobial effect close to that of the reference product was estimated at approximately 110 s for the mixed pathogen population and 120 s for donor dental plaque.

Table 3. Overall bacterial death rate constant, Koverall, s^{-1}.

Mouthwash	Mixed pathogen population	Donor dental plaque
Children's mouthwash - sample 1	0.132 ± 0.001b	0.123 ± 0.001b
Children's mouthwash - sample 0	0.075 ± 0.001c	0.084 ± 0.001c
Eludril classic®	0.151 ± 0.001a	0.148 ± 0.001a

**Different superscript letters indicate statistically significant differences (Tukey HSD, $p < 0.05$).*

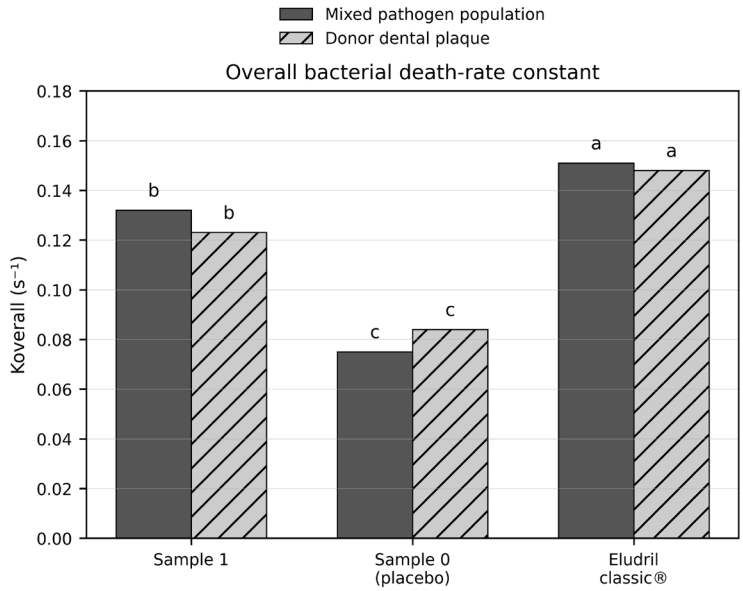


Fig. 4. Overall bacterial death rate constant (Koverall, s^{-1}) for tested mouthwash formulations.

Discussion of antimicrobial performance

The results of the present study confirm that the antimicrobial efficacy of mouthwash components is strongly strain-specific and depends both on the chemical mechanism of action and on the structural organization of the microbial community. The highest resistance was observed in Tannerella forsythia and Prevotella intermedia, which is consistent with their recognized role as key periodontal pathogens and their capacity to participate in stable anaerobic biofilms. Similar observations have been reported by Socransky and Haffajee [16].

By contrast, Fusobacterium nucleatum and Streptococcus salivarius demonstrated susceptibility to a broader spectrum of tested compounds. This may be related to their intermediate ecological role in biofilm organization and to a comparatively lower resistance to membrane-active agents. Chlorhexidine showed universal antimicrobial activity against all tested strains [8]. Cetylpyridinium chloride also demonstrated broad-spectrum activity [17], with recent evidence indicating comparable antiplaque efficacy to chlorhexidine with improved tolerability [18].

The additional components of the formulation showed limited but potentially complementary activity. Aloe vera and sodium fluoride demonstrated selective effects in the tested model, whereas poloxamer 407 and the other technological excipients showed weak and strain-specific activity. Aloe vera possesses anti-inflammatory and antimicrobial effects [19]. Recent studies also support the antimicrobial potential of plant-derived formulations, including herbal mouthwashes [20].

An important finding is that the multicomponent prototype formulation demonstrated substantial antimicrobial activity, approaching that of Eludril classic® under short-exposure conditions. This suggests synergistic interactions between CPC, sodium fluoride, inulin and plant-derived compounds [21].

The direct-contact experiments are particularly relevant because they approximate the short exposure time occurring during mouth rinsing. The observed reduced efficacy in dental plaque confirms the increased resistance of biofilm-associated microorganisms [22].

Kinetic analysis further confirmed strong bactericidal activity of the prototype formulation. Although slightly slower than chlorhexidine, prolonged exposure could compensate for this difference, which is clinically relevant in pediatric patients [23].

The absence of chlorhexidine represents a major advantage. Contemporary research emphasizes microbiome-friendly antimicrobial strategies that preserve ecological balance while reducing pathogenic load [24]. Taken together, the results support the potential of the tested formulation as a clinically relevant alternative in pediatric preventive dentistry.

CONCLUSIONS

- The tested multicomponent prototype mouthwash exhibited significant antimicrobial activity against both planktonic and biofilm-associated oral microorganisms.
- The antimicrobial efficacy of the prototype formulation approached that of the chlorhexidine-containing reference product and remained substantial under short-exposure conditions.
- The observed antimicrobial performance is likely related to the combined effects of cetylpyridinium chloride, sodium fluoride and bioactive plant-derived components.
- The absence of chlorhexidine may represent an advantage in terms of safety and tolerability, particularly for repeated use in pediatric populations.
- Future studies should focus on long-term clinical evaluation, optimization of exposure time and assessment of the formulation effect on oral microbiome balance.

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Authors' contributions

T.Z., A.B.: conceptualization; T.Z., B.G., I.D., A.B.: methodology; B.G., I.D.: laboratory investigation; T.Z., B.G., I.D., A.B.: data acquisition and interpretation of microbiological results; T.Z., T.N.: writing - original draft preparation; T.Z., T.N., A.B.: writing - review and editing; A.B.: supervision. All authors have read and approved the final version of the manuscript.

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