Journal of Dentistry 165 (2026) 106318

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

In vitro antibiofilm and anti-caries effects of polyhexamethylene biguanide under a microcosm biofilm model

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ARTICLE INFO

Keywords:
Dental caries
Biofilm
Polyhexamethylene biguanide
Chlorhexidine

ABSTRACT

Objective: To evaluate the *in vitro* antimicrobial effect of polyhexamethylene biguanide (PHMB) solution compared to chlorhexidine digluconate (CHX) on the viability and vitality of microcosm biofilm. Material and methods: Biofilm was produced from the saliva of 3 volunteers, under 0.2 % sucrose exposure for 5 days. 180 enamel specimens were treated for 2 min with distilled water, 0.1 % PHMB, 0.2 % PHMB, 0.06 % CHX and 0.12 % CHX, once a day, for 5 days. The metabolic activity, viability of microorganisms and the vitality of biofilms were determined by resazurin, colony forming unit counts (CFU) and confocal scanning laser microscopy (CSLM), respectively (immediate and mediate analysis). Statistical analysis was conducted by Kruskal Wallis and Dunn's post-hoc tests ($\alpha = 0.05$). Results: Only CHX significantly reduced biofilm metabolic activity, with 0.06 % CHX showing an immediate effect and both CHX concentrations being effective in the mediate analysis. CFU analysis revealed that 0.06 % CHX had the strongest immediate antimicrobial effect against Lactobacillus sp. ($p = 0.0043$) and Streptococcus mutans ($p = 0.0159$), while PHMB showed no significant reductions in viable counts. Confocal microscopy demonstrated no immediate effects on whole-biofilm vitality; however, 0.2 % PHMB reduced vitality in the outer biofilm layer ($p = 0.0349$). PHMB further showed selective effects on biofilm structure, including an immediate reduction of β-polysaccharides ($p = 0.0442$) and live-cell volume ($p = 0.0259$), whereas CHX exerted more pronounced effects in the mediate analysis. Conclusions: PHMB demonstrated antibiofilm activity characterized by modulation of biofilm viability and extracellular matrix components, particularly in the immediate phase.

Introduction

The human oral microbiota comprises >700 species of bacteria and 23 fungal species, including opportunistic pathogens related to local and systemic diseases, such as periodontal alteration, cardiovascular and respiratory diseases, diabetes and osteoporosis [1,2]. The ethical difficulties related to *In vivo* studies have favored the development of *in vitro* laboratory models capable of mimicking the complexity of the oral microbiota [3]. The microcosm biofilm model is derived from a natural ecosystem capable of simulating the conditions of the mouth [4,5], reflecting the full complexity, diversity and heterogeneity of the *In vivo* environment, which allows it to be studied under controlled conditions [6,7]. In order to control bacterial growth and stabilize the oral microbiota, mouthwashes based on antimicrobial components are marketed, such as polyhexamethylene biguanide (PHMB) and chlorhexidine digluconate (CHX).

PHMB is a cationic polymer that interacts with acids and anionic molecules, such as the phospholipids in bacterial membranes [8,9]. It is chemically similar to CHX, consisting of between 2 and 40 biguanide units, whereas CHX contains only two [10,11]. The greater the number of polymer units, the stronger the binding and disruption of anionic phospholipids in the bacterial cell wall [12,13]. They have different terminal chains, which leads to differences in their therapeutic activity and toxicological profile [10]. PHMB has a broad spectrum of action, covering Gram-positive and Gram-negative bacteria (*Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis, Enterobacter cloacae* and

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https://doi.org/10.1016/j.jdent.2025.106318
Received 28 October 2025; Received in revised form 21 December 2025; Accepted 24 December 2025
Available online 25 December 2025

Streptococcus lactis), fungi (*Candida albicans, Aspergillusniger, Fusariumsolani* and *Saccharomycescerevisiae*), yeasts, protozoa (*Acanthamoeba spp*) and against the enveloped HIV virus [11,14]. It also enables selective binding and condensation to bacterial DNA, interrupting its cell division, which may explain the low risk of antimicrobial resistance [15].

Chlorhexidine digluconate (CHX) is the gold standard for dental biofilm control due to its substantivity [16], a characteristic also showed in the PHMB solution on oral mucosa and bacterial plaque [17]. Studies on the effectiveness of PHMB in the oral cavity and plaque inhibitory capacity are scarce [18]. *In vivo* studies have reported antibacterial effectiveness of 0.04 % PHMB on oral mucosa [18]. and 0.12 % PHMB on tooth surfaces [19], but both were less effective than CHX. PHMB was effective against monospecies biofilms of *S. sanguinis, C. albicans, F. nucleatum* and *S. aureus* resistant to CHX [20,21]. It is also possible to apply PHMB in higher concentrations due to its low toxicity and good biocompatibility compared to CHX [22]. In addition, it remains active when highly diluted, unlike CHX, which loses its efficacy when diluted to <10 % of its original [20].

Some studies have shown that 0.2 % PHMB solution has antimicrobial activity comparable to CHX and can be used as an alternative in the choice of mouthwashes [18,19,23]. Thus, it is necessary to investigate the effect of PHMB on polymicrobial biofilm models, specifically on the inhibition of bacterial growth and/or cellular metabolic inhibition, in addition to its action on biofilm structure [24]. The choice of mouthwash varies according to the initial oral diagnosis, based on knowledge of the substance's mechanism and period of action, and applicability based on the prevention and/or treatment of oral pathologies [25].

Therefore, the aim of this study was to evaluate *in vitro* the antimicrobial effect of PHMB on viability, vitality, metabolic activity, extracellular matrix and extracellular polysaccharides in a microcosm biofilm model, compared to CHX. The null hypothesis tested was that PHMB had no promising antimicrobial and anti-caries effects compared to CHX.

Materials and methods

Ethical aspects

This research was approved by the Human Research Ethics Committee (CAAE: 24,525,419.4.0000.5417) and by the Ethics Committee on the Use of Animals (CEUA: 032/2019).

Saliva collection

This experimental *in vitro* study was carried out by collecting saliva from 3 volunteers. The inclusion criteria were young adults aged between 25 and 35 years, adequate salivary flow (stimulated > 1 mL/min and non-stimulated > 0.3 mL/min) and absence of active caries lesions. The exclusion criteria were those diagnosed with syndromes and/or systemic diseases, the presence of caries lesions and the use of antibiotics in the last 90 days [26]. The saliva samples were filtered with glass wool and subsequently plated on Soy Triptone Blood Agar, Mitis Salivarius Agar, Mitis Salivarius Agar containing 0.2 % bacitracin and 20 % sucrose, Rogosa Agar with 0.13 % glacial acetic acid and Saboroud Agar. After 24 h, the Colony Forming Units (CFU) were counted, and the three most heterogeneous saliva samples were selected to be included in the bacterial pool.

Tooth sample preparation and sample size calculation

180 samples of 4 × 4 mm^2 bovine enamel were prepared from incisor teeth, using a semi-precision cutting machine. The surfaces were polished with water-cooled silicon carbide disks (600, 800 and 1200 grit of Al_2O_3; Buehler, Lake Bluff, USA) for 10 s and with felt paper wetted by diamond spray (1 μm; Buehler, Lake Bluff, USA). Finally, the samples were ultrasonically cleaned (T7 Thornton, Unique, São Paulo, Brazil) for 20 min and kept in sterile deionized water until use. The sample size was based on a previous study [27].

Treatment groups and analysis time

The PHMB and CHX solutions were used in commercially established concentrations (0.2 % PHMB, 0.06 % CHX, 0.12 % CHX), and non-commercial concentration (0.1 % PHMB), obtained from macrodilution with sterile distilled water in a 1:1 ratio. Therefore, the treatment groups were: 1) distilled water (negative control); 2) 0.1 % PHMB; 3) 0.2 % PHMB; 4) 0.06 % CHX and 5) 0.12 % CHX. The antimicrobial solutions were placed in direct contact with the biofilm for 2 min. The test was carried out in triplicate with two repetitions. For confocal analysis, aqueous control group (CG) was used as negative control.

The analyses were carried out at two different times: a) immediate analysis: a neutralizing solution (Tween) (3 g lecithin, 0.34 g K_2HPO_4, 30 g polysorbate, pH 7.2) was applied after the treatments for 2 min to remove the substantive effect of the solutions, and the laboratory analyses were carried out immediately [28]; b) mediate analysis: after treatment, the media was renewed and incubated for a further 24 h in a microaerophilic oven.

Microcosm biofilm formation

A modified version of the Active Attachment Biofilm Model Amsterdam (ACTA, Amsterdam, The Netherlands) was used [29], where the specimens were fixed with light condensation silicone (Zetaplus, Zhermack, Badia Polesine, Italy) and then sterilized. The bacterial inoculum was obtained by successive cultures of the bacterial pool over a period of 5 days. Bacterial growth was obtained in exponential phase, over a period of 24 h, in modified McBain medium (mucin at 2.5 g L^{-1}, peptone at 2.0 g L^{-1}, casein peptone at 2.0 g L^{-1}, yeast extract at 1.0 g L^{-1}, NaCl at 0.35 g L^{-1}, KCl at 0.2 g L^{-1}, $CaCl_2$ at 0.2 g L^{-1}, hemin at 0.001 g L^{-1}, vitamin K1 at 0.0002 g L^{-1}, PIPES buffering agent at 50 mmol L^{-1}, sucrose at 0.4 % diluted in deionized water/L, pH 7.0) [30], followed by incubation in an oven at 37 °C. Due to its photosensitivity, the medium was handled without being exposed to cold light. In Falcon tubes with McBain medium, 400 μL of the bacterial pool and 40 μL of 50 % sucrose were added, resulting in McBain medium at 0.4 % sucrose, vortexed and incubated in microaerophilia for 14 h - 16 h (overnight).

Culture plates containing enamel samples ($n = 9$ in 3 independent experiments) were filled with 750 μL of inoculum (bacterial pool) and incubated in 750 μL of sterile McBain medium at 37 °C for 5 days. Every 24 h, the medium was renewed with 1500 μL of McBain at 0.2 % sucrose. On the 5th day, the samples were washed with 1 mL of cysteine-peptone solution (CPS) to remove weakly adhered bacteria.

Colony-forming units (CFU) counting

The samples, in buffered peptone water (BPW) medium, were incubated in microaerophilia at 37 °C for 3 h, then dispersed by sonication on ice at 40 mW, 1 pulse s^{-1} for 1 min (Single Ultra-Sonic Cell Disruptor, Merse, Campinas, Brazil). Serial microdilution (10° to 10^{-4}) was then carried out in CPS medium. Aliquots of 25 μL of each dilution were inoculated onto Mitis Salivarius Agar containing 0.2 % bacitracin and 20 % sucrose and Rogosa Agar with 0.13 % glacial acetic acid to determine the counts of streptococcus mutans and total lactobacilli, respectively. All the plates were incubated for 48 h in microaerophilia at 37 °C and counted using optical microscopy.

Resazurin metabolism assay

The metabolic activity of the biofilm was determined according to Jiang et al. (2011) [31]. After each treatment, the samples were washed with 1 mL of buffered peptone water (PBS) (10 g peptone, 5 g NaCl, 3.5 g Na_2PO_4, 1.5 g KPO_4 per liter, pH 7.0), then stained with 1 mL of 0.0016

% resazurin solution and incubated in microaerophilia for 2 h, and the optical densities measured on a spectrophotometer (BioTekInstruments, Winooski, USA) at 608 nm. The procedure was repeated for each sample in both the immediate and mediate analyses, obtaining a total of 9 readings for each group.

Vital fluorescence microscopy evaluation

After being washed with CPS, the samples were stained with 20 µl of the LIFE/DEAD BacLight bacterial viability kit (Invitrogen Molecular Probes, Eugene, Oregon, USA), and kept in the dark for 7 min. They were then washed in CPS medium, stained with 20 µl of calcofluor solution and kept in dark conditions for 1 min [32] and washed again in deionized water. The absorption and emission wavelengths were 480/500 nm for the SYTO 9 dye and 490/635 nm for propidium iodide (SHEN, STOJICIC, HAAPASALO, 2010). In addition to these dyes, 20 ul of AlexaFluor was added to the samples 24 h before analysis by confocal microscopy to stain the extracellular polysaccharides [33].

On each sample, 3 different fields were viewed using an inverted laser scanning confocal microscope (Micro Systems Engineering GmbH, Mannheim, Germany), with 488 nm excitation, BP520 dual detection for LIVE/DEAD, 40x objective and NA 0.65. The fields were determined along the horizontal axis of the biofilm samples, starting from the edge of the glass blocks, at a distance of 100 µm. To compare the images, the laser power and aperture were kept constant. At each selected site, the entire depth was analyzed and recorded, based on the thickness of the biofilm. The images were corrected and synthesized in Leica LAS AF Lite software (Leica, Wetzlar, Germany) and analyzed to determine the vitality of intact biofilms by the ratio between the green signals and the sum of the green and red signals [34].

The images were presented individually in 2-D and reconstructed in 3-D projections. Vertical sections (xz) of 1 µm and side views of the reconstructed images were used to determine the thickness and architecture of the biofilm. The viability of bacterial cells at different depths of the biofilm was determined by averaging the pixel intensity of the green (live bacteria) and red (dead bacteria) signals, calculated using Leica QWIN Image Analysis software (Leica, Wetzlar, Germany). The fluorescence emitted by the intensity of pink and blue pixels determined the percentage of extracellular matrix and extracellular polysaccharides, respectively, calculated by software [33]. The ratio of green signals to the sum of green and red signals was calculated and determined as the percentage of viable cells.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 10 (GraphPad Software, Boston, USA). The CFU counts were log10 transformed before the statistical tests were applied. The hypothesis of normal distribution of the data obtained was rejected by the Kolmogorov-Smirnov test. The Kruskal-Wallis non-parametric test for independent samples and Dunn's post-hoc test were used to determine significant differences between the specific groups ($p < 0.05$).

Results

CFU counting

Regarding viable plate counts, in the immediate analysis, 0.06 % CHX ($p = 0.0043$) showed the best antimicrobial effect compared with negative control for *Lactobacillus sp*. (Table 1). Moreover, 0.06 % CHX ($p = 0.0159$) was able to significantly reduce the number of *Streptococcus mutans* compared with negative control.

In the mediate analysis, only 0.06 % CHX ($p = 0.0108$) and 0.12 % CHX ($p < 0.0001$) showed a protective effect against total lactobacilli compared to the control group. For *S. mutans*, none of the treatments were able to protect the enamel surface.

Metabolic activity of the biofilm

In the immediate analysis, only CHX 0.06 % significantly reduced the metabolic activity of the biofilm when compared with the negative control ($p = 0.0167$) (Table 2). For the mediate analysis, both concentrations of CHX (0.06 % and 0.12 %) were effective compared with the negative control ($p = 0.016$ and $p < 0.0001$, respectively). No statistically significant difference was observed between the PHMB concentrations and the control group ($p > 0.05$).

Confocal scanning laser microscope assay

Vitality of different layers and whole biofilm

Biofilm vitality by CSLM was analyzed for the entire biofilm and in three different layers: outer, middle and inner. In the immediate analysis, expect to 0.2 % PHMB in the outer layer ($p = 0.0349$), no significant difference was observed between the treatment solutions and the control group in the whole biofilm, the middle and inner layers (Table 3).

For mediate analysis, only the commercial solution 0.12 % CHX statistically decreased the viability of the whole biofilm ($p = 0.0305$). On the outer and middle layer, no solution showed a protective effect. With regard to the inner layers, only 0.12 % CHX was effective ($p < 0.0001$).

Table 2
Metabolic activity of the biofilm. Data (absorbance) are presented as median with 25th and 75th percentiles. Statistically significant differences within each column are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

Treatment	Immediate	Mediate
Control	97.9 (83; 120.7)^{A}	99.6 (89.43; 111.2)^{A}
0.06 % CHX	33.5 (19.5; 104.3)^{B}	26.9 (6.3; 59.5)^{BC}
0.12 % CHX	78.4 (47.9; 96.4)^{AB}	5.9 (4.8; 44.7)^{C}
0.1 % PHMB	81.5 (59.3; 91.6)^{AB}	81.8 (60.7; 119.8)^{AB}
0.2 % PHMB	81.4 (72.8; 98.3)^{AB}	87.8 (59.6; 118.2)^{AB}

Table 1
Colony-forming units of the different microbial species. Data (log 10/mL) are presented as median with 25th and 75th percentiles. Statistically significant differences within each row are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

Species	Analysis time	Control	0.06 % CHX	0.12 % CHX	0.1 % PHMB	0.2 % PHMB
Total Lactobacillus	Immediate	5.8 (5.0; 6.3)^{A}	4.4 (3.9; 4.8)^{B}	5.2 (4.5; 5.8)^{AB}	5.0 (4.7; 5.2)^{AB}	4.9 (4.4; 5.6)^{AB}
	Mediate	5.5 (5.3; 6.0)^{A}	3.9 (3.6; 4.2)^{BC}	3.5 (3.2; 3.7)^{C}	4.5 (3.5; 5.9)^{AC}	4.9 (4.2; 5.7)^{AB}
Total Streptococcus	Immediate	7.1 (5.5; 7.6)^{A}	5.3 (3.8; 5.9)^{A}	5.9 (5.4; 6.6)^{A}	6.3 (4.3; 6.6)^{A}	6.4 (5.1; 7.7)^{A}
	Mediate	6.6 (6.0; 7.5)^{A}	6.6 (6.0; 7.1)^{A}	6.5 (6.1; 8.0)^{A}	6.3 (6.0; 6.5)^{A}	6.4 (6.3; 8.1)^{A}
Streptococcus mutans	Immediate	7.4 (6.1; 7.4)^{A}	5.1 (3.7; 5.9)^{B}	6.0 (4.7; 6.6)^{AB}	5.7 (4.1; 6.5)^{AB}	6.3 (5.0; 7.7)^{AB}
	Mediate	5.6 (5.4; 7.1)^{A}	6.4 (5.9; 7.0)^{A}	6.0 (4.6; 8.0)^{A}	6.2 (3.9; 6.4)^{A}	6.2 (4.7; 8.0)^{A}
Total microorganisms	Immediate	7.1 (5.6; 7.6)^{A}	5.3 (4.3; 5.8)^{A}	6.0 (5.4; 6.7)^{A}	6.2 (5.0; 6.5)^{A}	6.4 (5.3; 7.7)^{A}
	Mediate	6.6 (6.2; 7.4)^{A}	6.5 (6.4; 7.5)^{A}	6.3 (6.0; 7.9)^{A}	6.5 (6.3; 7.0)^{A}	6.5 (5.9; 8.0)^{A}
Candida albicans	Immediate	6.8 (5.5; 7.6)^{A}	5.3 (4.0; 5.6)^{A}	6.2 (5.4; 6.7)^{A}	6.2 (4.9; 6.8)^{A}	6.5 (5.2; 7.7)^{A}
	Mediate	6.6 (6.0; 7.4)^{A}	6.5 (6.3; 6.5)^{A}	6.1 (6.1; 8.1)^{A}	6.4 (6.1; 8.1)^{A}	7.6 (6.0; 8.0)^{A}

Table 3
Vitality of the entire biofilm or different layers. Data (percentage) are presented as median with 25th and 75th percentiles. Statistically significant differences within each row are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

Layer	Analysis time	Control	0.06 % CHX	0.12 % CHX	0.1 % PHMB	0.2 % PHMB
General	Immediate	29.0 (11.8; 50.4)^{A}	23.5 (13.1; 32.1)^{A}	18.6 (8.6; 40)^{A}	9 (3.7; 26.8)^{A}	14.6 (6.5; 27)^{A}
	Mediate	23.5 (13.4; 45.9)^{A}	18.9 (15.2; 28.3)^{AB}	14.1 (6.7; 22.0)^{B}	25 (17.1; 31.9)^{A}	24.3 (16.8; 39.4)^{A}
Outer	Immediate	16.8 (12.4; 24.8)^{B}	17.7 (12.3; 23.9)^{B}	25.7 (13.7; 33.2)^{AB}	12.5 (2.6; 20.4)^{B}	34 (21.5; 66.7)^{A}
	Mediate	26.7 (18.1; 40.2)^{AB}	20.8 (14.7; 28.6)^{B}	23.5 (15.4; 34.4)^{AB}	31.8 (20.4; 36.7)^{AB}	30.8 (21.1; 43.8)^{A}
Middle	Immediate	29 (11.4; 42.2)^{A}	16.7 (14; 27.1)^{A}	25.4 (16.4; 34.2)^{A}	14.9 (7.4; 31.9)^{A}	21.6 (10.9; 32.2)^{A}
	Mediate	26 (16.8; 49.2)^{A}	20.5 (17.1; 28.6)^{A}	16.2 (12.9; 30.9)^{A}	31.8 (23.4; 38)^{A}	37 (20; 45.7)^{A}
Inner	Immediate	29.4 (17.4; 48)^{A}	19.8 (13; 26.3)^{A}	16.8 (10.6; 29.8)^{A}	14.7 (4.8; 36.2)^{A}	21.6 (7.6; 49)^{A}
	Mediate	34.7 (18.9; 49.6)^{A}	20.1 (15.6; 28.8)^{AB}	13.6 (6.7; 19.7)^{B}	30 (22.6; 37.2)^{A}	22.5 (16.1; 36.1)^{A}

Live and dead cells

In the immediate analysis, only 0.1 % PHMB significantly reduced the live cells of the biofilm when compared with the negative control ($p = 0.0259$), showing better performance than CHX (0.06 % and 0.12 %) (Table 4). On the other hand, regarding dead cells, none of the solutions treatment were effective.

However, in the mediate analysis, only 0.06 % CHX was effective in reducing the number of live cells compared with the negative control ($p = 0.0674$). 0.12 % CHX did not differ significantly from the control group ($p > 0.9999$). With regard to dead cells, 0.06 % CHX showed the best protective effect ($p = 0.0024$) followed by 0.12 % CHX and 0.1 % PHMB in which there was no statistical difference.

Extracellular matrix

In the immediate analysis, only 0.2 % PHMB caused a dramatic reduction of β-polysaccharides ($p = 0.0442$), showing higher effect than the gold standard (0.06 % and 0.12 %) (Table 5). In the mediate analysis, no solution reduced the β-polissaccharides compared to negative control ($p = 0.472$).

Table 4
Live and dead biofilm volume. Data (10^{-3} μm^3) are presented as median with 25th and 75th percentiles. Statistically significant differences within each column are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

	Live		Dead	
Treatment	Immediate	Mediate	Immediate	Mediate
Control	40.2 (16.9; 179.9)^{B}	35.8 (17.3; 67.7)^{AB}	144.3 (64.8; 311.5)^{A}	111.5 (63.5; 291.6)^{B}
0.06 % CHX	57.8 (17.5; 117.7)^{AB}	87.8 (51.5; 132)^{A}	203.7 (105; 356.9)^{A}	435.2 (251.7; 581.9)^{A}
0.12 % CHX	38.7 (16.7; 91.2)^{AB}	35.9 (9; 69.5)^{B}	150 (65.4; 350.4)^{A}	259.5 (61.9; 666.1)^{AB}
0.1 % PHMB	16.1 (4.4; 27.7)^{A}	79.3 (30.5; 200)^{A}	132.4 (32.7; 370.4)^{A}	308.2 (128.6; 450.4)^{AB}
0.2 % PHMB	27 (4.9; 53)^{AB}	65.3 (34.7; 131.4)^{AB}	81.5 (26.5; 475.6)^{A}	213.4 (101.2; 439.1)^{AB}

Table 5
Extracellular matrix. Data (10^{-3} μm^3) are presented as median with 25th and 75th percentiles. Statistically significant differences within each column are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

Treatment	Immediate	Mediate
Control	148.1 (41.6; 429.5)^{A}	209 (100.6; 399.5)^{AB}
0.06 % CHX	109.1 (28.3; 167.2)^{AB}	289 (170; 442.5)^{A}
0.12 % CHX	91.1 (32.2; 174.1)^{AB}	115.5 (42.6; 261.3)^{B}
0.1 % PHMB	46.5 (11.5; 108.6)^{BC}	126.7 (49.1; 310.4)^{AB}
0.2 % PHMB	15.6 (4.8; 57.6)^{C}	228 (55; 427.4)^{AB}

Extracellular polysaccharides

In the immediate analysis, none of the treatments were effective in reducing extracellular polysaccharides (Table 6). On the other hand, in the mediate analysis, 0.06 % CHX was effective in reducing polysaccharides ($p = 0.0001$), followed by 0.1 % PHMB ($p = 0.0171$).

Discussion

The null hypothesis that PHMB would not present promising antimicrobial and anti-caries effects compared to CHX was partially rejected. PHMB exhibited selective and immediate anti-biofilm effects in confocal microscopy analyses, including reductions in outer-layer viability, live cell counts, β-polysaccharide content, and extracellular polysaccharides, with some concentrations outperforming CHX. These findings indicate that PHMB demonstrated short-term and structure-related anti-caries potential, particularly targeting extracellular matrix components and superficial biofilm layers.

To the best of our knowledge, this is the first *in vitro* microcosm biofilm study associated with PHMB treatment, mimicking the oral environment, reproducing the conditions of a supragingival biofilm, and presents complex microbial communities. It can be produced in the laboratory (controlled environment), reducing the possibility of bias [35]. Using this model, it was evaluated the protective effect of different concentrations of CHX and PHMB in solution form, during the formation of the bacterial biofilm for 5 days, on the bacteria metabolism, vitality of the biofilm and components of the extracellular matrix.

The superior antimicrobial performance of chlorhexidine (CHX) observed in CFU counting and metabolic activity assays is consistent with its well-documented bactericidal and bacteriostatic properties against cariogenic microorganisms. Previous studies have demonstrated that CHX significantly reduces viable counts of Streptococcus mutans and Lactobacillus spp. in both planktonic cultures and biofilm models, mainly due to its ability to disrupt bacterial membranes and precipitate cytoplasmic proteins [35–38]. In particular, the sustained antimicrobial effect of CHX observed in the mediate analysis corroborates its known substantivity, which allows prolonged retention on oral surfaces and continued antimicrobial action [39–42].

The lack of protective effects against S. mutans in the mediate analysis in the present study is in agreement with previous report indicating that mature cariogenic biofilms exhibit increased resistance to antimicrobial agents, even those considered gold standards [43]. This

Table 6
Extracellular polysaccharides. Data (10^{-3} μm^3) are presented as median with 25th and 75th percentiles. Statistically significant differences within each column are indicated by different superscript letters. CHX = chlorhexidine; PHMB = polyhexamethylene biguanide.

Treatment	Immediate	Mediate
Control	44.5 (17.8; 278.5)^{A}	60 (31.1; 106.2)^{B}
0.06 % CHX	53.4 (29.9; 103.1)^{A}	241.5 (145; 353.9)^{A}
0.12 % CHX	84.6 (27.3; 141)^{A}	105.5 (36; 408)^{AB}
0.1 % PHMB	32.8 (23.5; 60)^{A}	166 (75.2; 329)^{A}
0.2 % PHMB	33.3 (14.4; 131.3)^{A}	85 (61.7; 181.1)^{AB}

resistance has been attributed to biofilm thickness, reduced diffusion, and the protective role of the extracellular matrix [44], which may explain why none of the tested agents were able to fully prevent recolonization on enamel surfaces.

The metabolic activity findings further corroborate earlier observations that CHX interferes not only with bacterial viability but also with enzymatic activity and energy metabolism within biofilms [37]. In contrast, the absence of significant metabolic suppression by PHMB aligns with studies suggesting that PHMB primarily exerts membrane-active and structural effects rather than deep metabolic inhibition [45,46]. Resazurin method is an effective alternative for viewing the reading of tests on microplates. This compound works as a chromogenic substrate for dehydrogenase enzymes, acts as an oxy-reduction indicator, and is reduced (by hydrogen gain) by flavins linked to enzymes related to the transport system during cellular metabolism [47]. However, in the evaluated concentrations, PHMB could not reduce the metabolic activity of the biofilm compared to the negative control. Seems the antimicrobial effect of PHMB on microcosm biofilm is strain-dependent, may indicate that the assay is less sensitive in detecting single log-based differences between treatment groups. Intrinsic resistance to CHX is known from bacterial spores and mycobacteria and is due to their outer cell layers which form an impermeable barrier to the ingress of CHX molecules [48]. However, it is well known that cariogenic biofilms represents a complex polymicrobial 3-dimensional structure composed by both gram-positive and negative bacteria, meshed in an extracellular matrix adhered to the enamel surface and capable of penetrating the interior of dentin. These outcomes cannot be compared with previous studies that tested the effect of PHMB in monospecies or duospecies biofilm and not in a complex microorganism environment as shown in the present study, since diverse factors as microbial loads and substrate of biofilm growth affect the susceptibility of microorganisms to treatments.

Confocal laser scanning microscopy provided important insights into the layer-specific effects of PHMB. The selective reduction of viability in the outer biofilm layer observed immediately after PHMB exposure corroborates previous reports describing PHMB as a polycationic polymer that preferentially interacts with negatively charged bacterial membranes at the biofilm surface [46]. In contrast, the ability of CHX to significantly affect the inner biofilm layers in the mediate analysis confirms its superior penetration and cumulative antimicrobial action over time, as previously demonstrated in mature oral biofilms.

The discrepancy between CFU counting and live/dead staining observed for PHMB has also been reported in the literature. Studies using fluorescence-based techniques have shown that membrane damage and loss of viability markers do not always correspond to immediate loss of cultivability, particularly for biofilm-associated bacteria [49,50]. This supports the interpretation that PHMB induces early membrane perturbation that may precede irreversible cell death.

One of the most relevant contributions of the present study is the pronounced immediate reduction of β-polysaccharides induced by PHMB, exceeding the effects of CHX. This finding is supported by previous studies emphasizing that biguanide polymers can interfere with extracellular polymeric substances by destabilizing electrostatic interactions within the biofilm matrix [51]. Koo et al. (2013) highlighted that targeting extracellular polysaccharides is a key strategy for reducing biofilm virulence, reinforcing the significance of the matrix-modulating effects observed for PHMB [44].

However, the absence of sustained matrix reduction in the mediate analysis contrasts with reports showing prolonged suppression of extracellular polysaccharides by CHX [52], suggesting that PHMB-induced matrix disruption may be transient or compensated by biofilm remodeling. The delayed reduction of extracellular polysaccharides observed for CHX in the present study is consistent with these previous findings and may result from indirect effects associated with reduced bacterial activity and glucosyltransferase inhibition.

A possible limitation of our study may be related to saliva collection. Instead of using saliva from healthy individuals, collecting saliva from volunteers with active caries might better mimic clinical practice. Another important aspect is that, in future studies, progressive comparison parameters could be adopted during the treatment period to determine when PHMB may lose its protective effect on the biofilm. While CHX showed superior and more sustained antimicrobial effects, the potential benefit of PHMB lies in its distinct mode of action on biofilm organization rather than broad-spectrum bactericidal activity. Additionally, PHMB is known for its lower propensity to induce adverse effects commonly associated with long-term CHX use, such as tooth staining, taste alteration, and mucosal irritation. Therefore, PHMB may represent a complementary or adjunctive agent aimed at biofilm modulation, particularly in preventive strategies where prolonged use and patient compliance are critical.

This study provided a comprehensive and multi-parametric evaluation of PHMB in comparison with CHX, integrating microbiological, metabolic, and structural biofilm analyses under immediate and mediate conditions. Unlike previous studies that primarily focused on planktonic bacteria or single biofilm endpoints, the present investigation adopted microcosm biofilm model to enable the complex interactions between the microorganisms. Additionally, by combining CFU counting, metabolic activity assays, and confocal laser scanning microscopy with layer-specific and extracellular matrix analyses [53], this study advances current knowledge by revealing that antimicrobial efficacy and biofilm modulation are not necessarily overlapping phenomena.

Within the limitations of this study, PHMB demonstrated a distinct pattern of action, characterized by transient and structure-oriented effects on the biofilm. Rather than exerting prolonged antimicrobial activity, PHMB primarily affected superficial biofilm organization and matrix-related components, suggesting an alternative mechanism of action. This behavior highlights the relevance of targeting biofilm architecture as an adjunctive strategy in caries control and supports further investigation into PHMB as a biofilm-modulating agent. Further studies are required to evaluate its long-term efficacy, optimal concentration, and clinical relevance.

CRediT authorship contribution statement

Caroline Gomes Carvalho: Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **João Victor Frazão Câmara:** Writing – original draft, Resources, Formal analysis. **Daniela Alejandra Cusicanqui Méndez:** Methodology, Investigation, Formal analysis, Data curation. **Anton Schestakow:** Formal analysis, Software, Writing – review & editing. **Thiago Cruvinel:** Validation, Resources, Investigation, Formal analysis, Data curation. **Paulo Sergio da Silva Santos:** Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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.

Acknowledgements

This study was financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – 001.

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