Anti-biofilm activity of oral healthcare products containing chlorhexidine digluconate and Citrox®

Jenaniy Jeyakumar[1], Anton Sculean[2], Sigrun Eick[3]

Purpose: To analyze in vitro new formulations with Citrox and chlorhexidine digluconate (CHX) regarding their antibacterial activity against planktonic bacteria and their potential to inhibit biofilm formation or to act on existing biofilms.

Materials and Methods: Five oral health care products with 0.05%–0.5% CHX formulations (four rinses and one gel) were compared with Citrox preparations and additive-free CHX solutions. The minimal inhibitory concentrations (MIC) were determined against 13 oral bacteria associated with caries or periodontitis. Further, the activity on retarding biofilm formation and on existing biofilms was analyzed; both a 'cariogenic' (5 species) and a 'periodontal' (12 species) biofilm were included.

Results: The MIC values did not differ between the CHX mouthrinse/gel formulations and the respective additive-free CHX solutions. Citrox was active against selected periodontopathogens (e.g. *Porphyromonas gingivalis*). The CHX formulations more effectively retarded biofilm formation than did solutions with the same concentration of CHX but without additives. The anti-biofilm activities depended on the CHX concentration in the formulations. Both CHX solutions and formulations (rinse and gel) were only slightly active on an already formed biofilm. Citrox did not exert any anti-biofilm effect.

Conclusion: The present in-vitro data support the anti-biofilm activity of the novel CHX, Citrox, poly-L-lysine and xylitol oral health-care formulations containing oral health care products. Further studies are warranted to confirm the present findings in various clinical settings.

Keywords: chlorhexidine digluconate, mouth rinse, periodontitis, caries, biofilm

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Introduction

Oral health-care products are widely used in prevention and therapy of biofilm-caused oral diseases. Among the antiseptics, products containing chlorhexidine digluconate (CHX) formulations are still the gold standard.[1] As recently reviewed, the beneficial effects of CHX are confirmed for reducing plaque accumulation, in dental caries, gingivitis, periodontitis.[2] Adjunctive use of CHX mouth rinses in non-surgical periodontal therapy results in additional probing depth reduction.[3] Using 0.12% CHX solution is recommended for high-caries-risk patients.[4] During fixed orthodontic therapy CHX varnishes are effective in reducing caries incidence.[5]

However, the CHX formulations have different side-effects e.g. extrinsic tooth staining, taste alterations, burning sensations.[6] To limit side-effects, CHX formulations may contain additives. In part, these additives interfere with the action of CHX. Certain in-vitro studies have reported that CHX mouth rinses containing an anti-discoloration system (ADS) were less active in comparison to other CHX preparations against planktonic bacteria[7] as well as when exposed to a growing biofilm.[8] In an vivo study, three 0.2% CHX formulation were compared: one with ADS, one with ethanol and one without ADS and ethanol. The formulation with ADS was less effective in plaque reduction and the

one with ethanol was less effective in reducing gingival inflammation .[9]

In several in-vitro-studies the cytotoxicity of CHX has been demonstrated.[10,11] The toxicity clearly depends on the concentration. Human fibroblasts and osteoblasts tolerate concentrations less than 0.02%[10] whereas 0.2% CHX showed a strong and 0.05% CHX a moderate cytotoxicity against gingival fibroblasts .[11] Thus, due to the reported adverse effects and the potential cytotoxicity, there is a need to develop formulations free of or containing a reduced concentration of CHX that might be equally effective as solutions containing 0.12% or 0.2% of CHX. Citrox® was proposed as a potential alternative or supplement. It is derived from citrus fruits, contains many different bioflavonoids and is used as an additive to commercial sanitizers[2] or in food products.[13]

In the present study, different new formulations with Citrox® and CHX in a concentration from 0.05% to 0.2% CHX were evaluated in vitro regarding their antibacterial activity against planktonic bacteria and their potential to inhibit biofilm formation or to act on existing biofilms. The biofilms included bacteria associated either with caries or with periodontal disease. The question to be answered was whether these formulations are equally or even more active as a solution with the same % of CHX and without additives.

Material and Methods

CHX formulations

In the experiment five oral health care products with CHX, four rinsing formulations and one gel (all obtained from CURADEN AG, Kriens, Switzerland) were included. The mouthrinsing formulations contained 0.2% CHX (CHX0.2C, Curaprox PerioPlus forte®), 0.12% CHX (CHX0.12C; Curaprox PerioPlus Protect®), 0.09% CHX (CHX0.09C, Curaprox PerioPlus Regenerate®) and 0.05% CHX (CHX0.05C; Curaprox PerioPlus Balance®). A gel formulation with 0.5 CHX (CHX0.5Cg) completed the tested oral health care products. Besides CHX, Citrox® and poly-L-lysine were constituents of all the formulations. Further, all the oral health care products contained xylitol and PVP-VA. Hyaluronic acid and cyclodextrin had been added to the CHX0.09C formulation, the CHX0.05C formulation was supplemented with sodium fluoride and the CHX0.5Citgel with hyaluronic acid.

As controls, two Citrox® preparations one without (Cit) and one with poly-L-lysine (CitPLL) were used. The negative control was 0.9% w/v NaCl solution and the positive controls were CHX solutions without additives in three CHX concentrations (0.2% (CHX0.2); 0.12% (CHX0.12) and 0.05 % (CHX0.05)).

Microorganisms

Fifteen different bacterial strains were used in the experiments:

- *Streptococcus gordonii* ATCC 10558
- *Actinomyces naeslundii* ATCC 12104
- *S. mutans* ATCC 25175
- *S. sobrinus* ATCC 33478
- *Lactobacillus acidophilus* ATCC 11975
- *Fusobacterium nucleatum* ATCC 25586
- *Campylobacter rectus* ATCC 33238
- *Parvimonas micra* ATCC 33270
- *Eikenella corrodens* ATCC 23834
- *Prevotella intermedia* ATCC 25611
- *Capnocytophaga gingivalis* ATCC 33624
- *Porphyromonas gingivalis* ATCC 33277
- *Tannerella forsythia* ATCC 43037
- *Filifactor alocis* ATCC 33099
- *Treponema denticola* ATCC 35405.

Except for *F. alocis* and *T. denticola*, minimal inhibitory concentration (MIC) values of the formulations and controls were determined against all other strains. 'Cariogenic' biofilm was formed of all streptococcal strains, *A. naeslundii* ATCC 12104 and *L. acidophilus* ATCC 11975. The 'periodontal' biofilm consisted of *S. gordonii* ATCC 10558, *A. naeslundii* ATCC12104, *Fusobacterium nucleatum* ATCC 25586, *C. rectus* ATCC 33238, *P. micra* ATCC 33270, *E. corrodens* ATCC 23834, *P. intermedia* ATCC 25611, *C. gingivalis* ATCC 33624, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *F. alocis* ATCC 33099, and *T. denticola* ATCC 35405. The strains were passaged on tryptic-soy agar plates (Oxoid, Basingstoke, GB) with 5% sheep blood (and with 10 mg/l N-acetylic muramic acid for *T. forsythia*). *T. denticola* ATCC 35405 was maintained in modified mycoplasma broth (BD, Franklin Lake, NJ) enriched with 1 mg/ml glucose, 400 µg/ml niacinamide, 150 µg/ml spermine tetrahydrochloride, 20 µg/ml Na isobutyrate, 1 g/ml cysteine, and 5 µg/ml cocarboxylase. All chemicals were bought from Merck KGaA, Darmstadt, Germany. All the strains were cultured at 37°C, streptococci, *A. naeslundii* ATCC 12104 and *L. acidophilus* ATCC 11975 with 10% of CO2, the other strains under anaerobic conditions.

Determination of MIC

The microbroth dilution technique was used to determine MIC values. After subcultivation of bacterial strains and

purity checking, a defined inoculum was added to Wilkins-Chalgren broth (Oxoid) supplemented with 10 µg/ml ß-NAD and defined concentrations of the formulations (starting from 10% of the final formulations). After an incubation time of 42 h (18 h for aerobes), the growth of microbes was analyzed by visual checking of turbidity (and if necessary, by subcultivation). MIC represented the lowest concentration without visible turbidity.

These experiments were made in independent replicates.

Activity on biofilms

Two different experimental designs were set, (a) the application of mouthrinse after mechanical removal of biofilm to show the influence on the formation of biofilms and (b) if there was any effect on an already formed biofilm (established biofilm).

a: Activity on biofilm formation:

The formulations and solutions were diluted to a 10% concentration with dH2O. The wells of four 96-well plates were coated with 25 µl of test substances. After 30 min of incubation, 25 µl/well protein solution (1.5% bovine serum albumin in PBS) were added for another 30 min. Bacteria were suspended each in 0.9% w/v NaCl according to McFarland 0.5. The suspensions for the respective biofilms were then mixed together, each with one part of *S. gordonii* ATCC10558, two parts of *A. naeslundii* ATCC 12104 and four parts of each other's bacterial strain. Thereafter (time 0 h) 200 µl of bacterial suspension mixed with nutrient broth (Wilkins-Chalgren broth supplemented with 10 µg/ml ß-NAD (and with 10 mg/l N-acetylic muramic acid for the 'periodontal' biofilm) in a ratio (volume 1 : 9) were added. After 6 h and 24 h of incubation in the respective atmosphere (cariogenic biofilm with 10% of CO_2, 'periodontal' biofilm under anaerobic conditions), the nutrient broth was carefully removed and the biofilms were briefly washed with 0.9% w/v NaCl. Then biofilms (one 96-well-plate each at the designated time) were scraped from the surface and suspended in 0.9% w/v NaCl and, after making a dilution series, plated on tryptic-soy agar plates. After an incubation in the respective conditions, the counts of colony forming units (CFU) were recorded. At 24h from the third 96-well-plate, quantification of the biofilms was made after staining with crystal violet according to recently published protocols.[14] From the fourth plate, the metabolic activity of the biofilm suspension was assessed with using Alamar blue as a redox indicator.[15]

b: Established biofilm

In each experiment three 96-well plates were used. The wells of the 96-well plates were coated with 25 µl/well protein solution (1.5% bovine serum albumin in PBS) for 30 min. The bacteria/nutrient broth mixture was then prepared as described above and each 225 µl were pipetted per well. The plates were incubated in the respective atmosphere for 48 h. Subsequently, in the case of the periodontal biofilm, each 10 µl of *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35405 were added per well and these plates were incubated for another 36 h. At 48 h for the 'cariogenic' biofilm and at 3.5 days for the 'periodontal' biofilm, the meanwhile established biofilms were treated with 25 µl of the test substances for 1 min after removing nutrient broth and washing briefly. After 1 min, nutrient broth (225 µl) was added and the biofilms were incubated for 1h. Analysis was then made as described above, namely for the CFU counts, biofilm mass and metabolic activity.

Statistical analysis

Statistical analysis was conducted using SPSS 26.0 (IBM (IBM, Chicago, IL, USA). These biofilm experiments were performed in two independent experiments in each independent quadruplicate. CFU counts were recorded as log10 CFU. Statistical analysis was made by first using ANOVA. For statistical significance, the post-hoc Bonferroni test was added tor record results. In the graphs (Figures 1-4) each statistically significant differences vs the controls as well as between the CHX formulation and its respective solution (CHX0.2C vs. CHX0.2, CHX0.12C vs. CHX0.12 and CHX0.05C vs. CHX0.05) are given. A p-value of 0.05 was considered to be statistically significant.

Results

MIC values

Comparing the MIC values of the CHX mouth rinsing formulations with the respective CHX solutions, the difference did not exceed one stage. The only exception was *L. acidophilus* which was more susceptible to the CHX solutions than to the CHX formulations. The Citrox® preparations were most active against *P. gingivalis* ATCC 33277, and moderately antibacterial against *F. nucleatum* ATCC 25586, *P. micra* ATCC 33270, and *C. gingivalis* ATCC 33624. Against all other strains the MICs were 5% or higher of the Citrox® formulations. There was no difference whether poly-L-lysine was added or not (Table 1).

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Table 1
Minimal inhibitory concentrations of oral health care products and CHX solutions (MIC % of the respective formulation/solution; tested in the range of 0.16% - 10%)

Strain	CHX0.2C	CHX0.12C	CHX0.09C	CHX0.05C	CHX0.5Cg	Cit	CitPLL	CHX0.2	CHX0.12	CHX0.05
Streptococcus gordonii ATCC 10558	≤0.16	≤0.16	≤0.16	≤0.16	≤0.16	>10	>10	≤0.16	≤0.16	≤0.16
Actinomyces naeslundii ATCC 12104	≤0.16	≤0.16	≤0.16	0.31	≤0.16	>10	>10	≤0.16	0.31	0.63
S. mutans ATCC 25175	≤0.16	≤0.16	≤0.16	≤0.16	≤0.16	>10	>10	≤0.16	≤0.16	≤0.16
S. sobrinus ATCC 33478	≤0.16	≤0.16	≤0.16	≤0.16	≤0.16	>10	>10	≤0.16	≤0.16	≤0.16
Lactobacillus acidophilus ATCC 11975	1.25	1.25	1.25	10	≤0.16	>10	>10	≤0.16	≤0.16	≤0.16
Fusobacterium nucleatum ATCC 25586	≤0.16	≤0.16	≤0.16	0.31	≤0.16	1.25	1.25	≤0.16	≤0.16	0.31
Campylobacter rectus ATCC 33238	≤0.16	≤0.16	≤0.16	≤0.16	≤0.16	5	5	≤0.16	≤0.16	≤0.16
Parvimonas micra ATCC 33270	0.63	0.31	0.63	0.63	≤0.16	1.25	1.25	0.31	0.31	0.63
Eikenella corrodens ATCC 23834	≤0.16	≤0.16	≤0.16	0.31	≤0.16	10	10	≤0.16	≤0.16	≤0.16
Prevotella intermedia ATCC 25611	≤0.16	≤0.16	≤0.16	≤0.16	≤0.16	5	10	≤0.16	≤0.16	0.31
Capnocytophaga gingivalis ATCC 33624	0.31	0.63	0.63	1.25	≤0.16	1.25	1.25	≤0.16	0.63	1.25
Porphyromonas gingivalis ATCC 33277	≤0.16	≤0.16	≤0.16	0.31	≤0.16	0.63	0.63	≤0.16	≤0.16	≤0.16
Tannerella forsythia ATCC 43037	≤0.16	≤0.16	≤0.16	0.31	≤0.16	5	5	≤0.16	0.31	0.63

Activity of CHX formulations on biofilm formation

According to the protocol, the final concentration in the assay was 1% of the formulation.

In the case of the 'cariogenic' biofilm, all CHX containing formulations/solutions statistically significantly reduced the CFU counts vs. control at 6 h (each $p<0.001$). The highest reductions were seen for CHX0.2C both after 6 h (-2.45 log10 CFU) and 24 h (-2.24 log10 CFU) of biofilm formation. At 6 h, the CFU counts were lower for the mouth rinsing formulations (CHX0.2C, CHX0.12C and CHX0.05C) each in comparison with their respective CHX controls (CHX0.2, CHX0.12 and CHX0.05; $p<0.001$ each). It is of interest to note that the low concentrated formulations reduced the CFU counts more than the higher concentrated CHX solutions, i.e. CHX0.09C was more active than CHX0.12 (-1.13 log10, $p<0.001$) and even more than CHX0.2 (-0.56 log10, $p=0.001$). At 24 h, only the counts after applying CHX0.2C were less than those of the control ($p<0.001$). Here also the difference vs CHX0.2 was statistically significant ($p<0.001$). The Citrox® formulations did not affect the CFU counts at any time (Figure 1A).

The biofilm mass of the cariogenic biofilm after 24 h of formation clearly depended on the CHX concentration in the formulations/solutions. Differences were statistically significant for all CHX formulations and the CHX0.2 and CHX0.12 solutions vs. control (each $p<0.001$). The biofilm mass was lower after CHX0.05C than after CHX0.05 ($p<0.001$) (Figure 1B).

The metabolic activity was reduced only after applying CHX0.2C and CHX0.5Cg ($p<0.001$ vs. control) (Figure 1C).

In the case of the 'periodontal' biofilm all formulations/solutions containing ≥ 0.09% CHX statistically significantly reduced the CFU counts vs. control (each $p<0.001$) at 6 h and 24 h of biofilm formation. After 6 h, there was also a statistically significant difference for CHX0.05C vs. control ($p<0.001$). The highest reductions were seen for CHX0.2C after 6 h (-2.42 log10 CFU) and for CHX0.5Cg after 24 h (-4.16 log10 CFU) of biofilm formation. At 6 h, the CFU counts were lower for the mouthrinsing formulations CHX0.12C

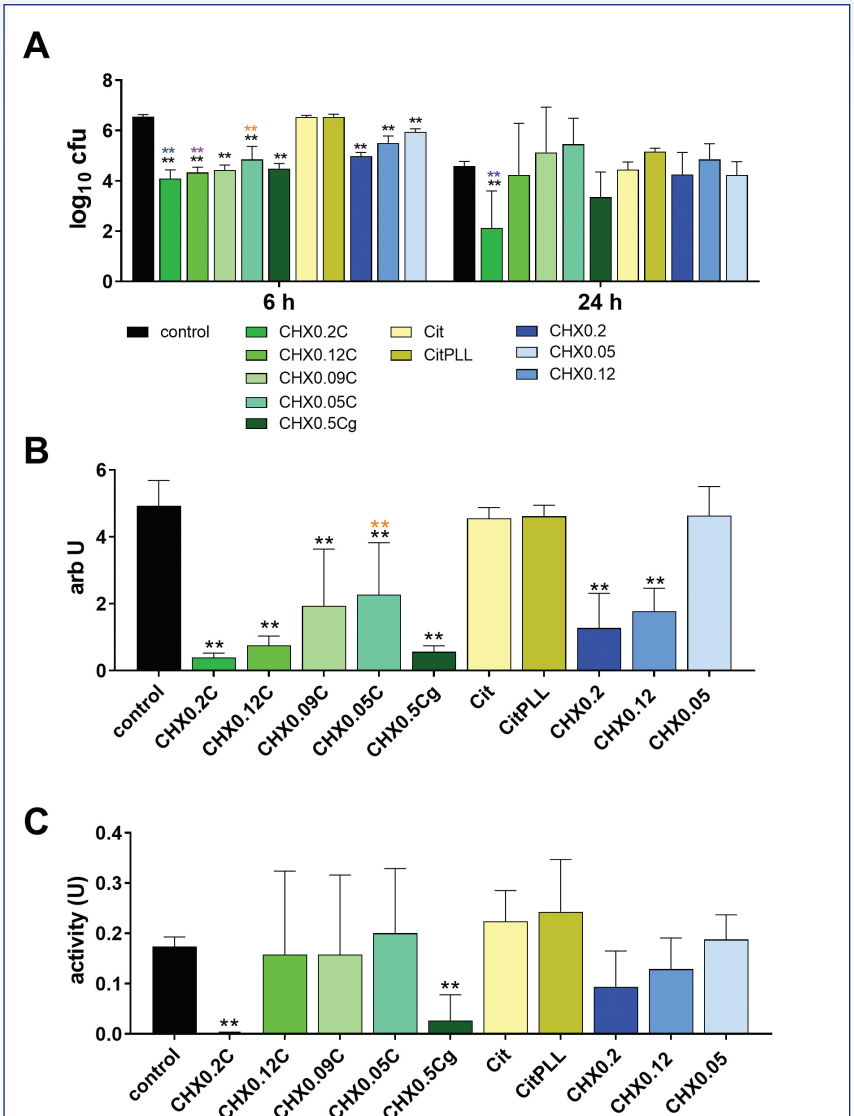

Figure 1: Activity of different formulations/ controls (coating of the surface with 10%, final concentration in the assays 1% of the formulations/ solutions) bacterial counts (A) after 6 h and 24 h of incubation, mass (B) and metabolic activity (C) both at 24 h in the formed "cariogenic" biofilm consisting of five different species. Tested formulations with CHX, Citrox® and poly-L-lysine: mouthrinsing formulations with 0.2% CHX (CHX0.2C), 0.12% CHX (CHX0.12C), 0.09% CHX (CHX0.09C) and 0.05% CHX (CHX0.05C) and a gel formulation with 0.5 CHX (CHX0.5Cg). Controls: 0.9% w/v NaCl as negative control (control), Citrox® preparations without (Cit) and with poly-L-lysine (CitPLL); CHX solutions without additives as positive controls with 0.2% CHX (CHX0.2), 0.12% CHX (CHX0.12) and 0.05 % CHX (CHX0.05).

and CHX0.05C in comparison with their respective control solutions CHX0.12 (p=0.001) and CHX0.05 (p=0.019). At 24 h the counts after applying CHX0.2C and CHX 0.12C were lower than those of the solutions CHX0.2 and CHX0.12 (each p<0.001) and those after CHX0.09C were reduced more than after CHX0.12 (p<0.001). The Citrox® formulations did not affect the CFU counts (Figure 2A).

The biofilm mass of the 'periodontal' biofilm after 24 h was lower after applying any of the CHX formulations or CHX0.2 and CHX0.12 (each p<0.001). CHX0.05C reduced the biofilm mass more than CHX0.05 (p<0.001) (Figure 2B).

The metabolic activity was reduced after applying CHX0.2C, CHX0.12C, CHX0.5Cg and CHX0.2 and CHX0.12 (each p<0.001 vs. control). It was increased after applying CHX0.05C (p<0.001) (Figure 2C).

Activity of CHX formulations on established biofilm

Differences between the two biofilm models were visible.

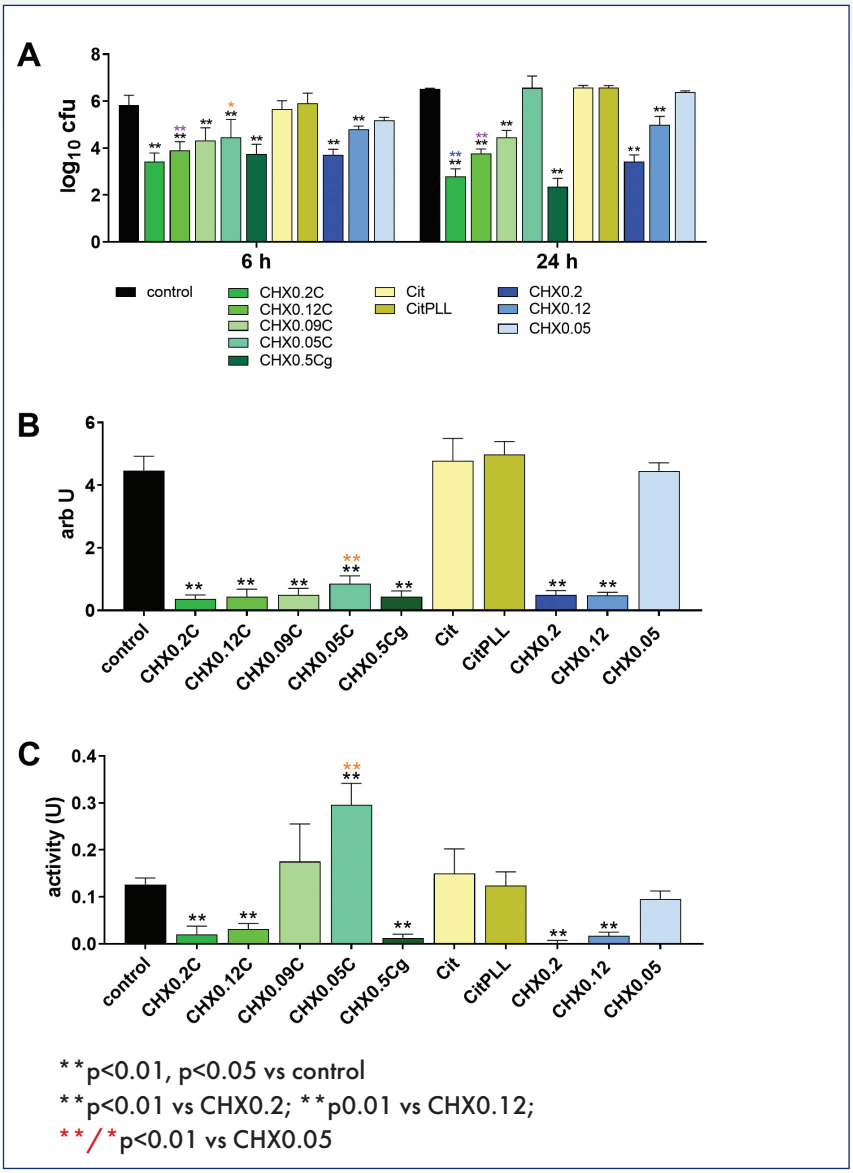

Figure 2: Activity of different formulations / controls (coating of the surface with 10%, final concentration in the assays 1% of the formulations/ solutions) bacterial counts (A) after 6 h and 24 h of incubation, mass (B) and metabolic activity (C) both at 24 h in the formed "periodontal" biofilm consisting of 12 different species
Tested formulations with CHX, Citrox® and poly-L-lysine: mouthrinsing formulations with 0.2% CHX (CHX0.2C), 0.12% CHX (CHX0.12C), 0.09% CHX (CHX0.09C) and 0.05% CHX (CHX0.05C) and a gel formulation with 0.5 CHX (CHX0.5Cg)
Controls: 0.9% w/v NaCl as negative control (control), Citrox® preparations without (Cit) and with poly-L-lysine (CitPLL); CHX solutions without additives as positive controls with 0.2% CHX (CHX0.2), 0.12% CHX (CHX0.12) and 0.05 % CHX (CHX0.05).

The controls of the cariogenic biofilm contained a mean of 5.26 log10 CFU, those of the 'periodontal' biofilm 7.22 log10.

In the 'cariogenic' biofilm CHX mouthrinsing formulations/ solutions with ≥0.09% CHX reduced the CFU counts (CHX0.2C, CHX0.12C p<0.001, CHX0.09C p=0.008, CHX0.2 p=0.001, CHX0.12 p=0.019). CHX0.2C was the most active, as no CFU were counted after application. The difference to CHX0.2 was statistically significant (p=0.001). The Citrox® formulations without CHX did not affect the CFU counts (Figure 3A). An influence on biofilm mass was not found for any of the formulations and controls (Figure 3B). Metabolic activity decreased after the application of CHX0.2C (p=0.009), CHX0.12C (p=0.002) and CHX0.2 (p<0.001) (Figure 3C).

In the 'periodontal' biofilm only the CHX mouth rinsing formulation/solution with 0.2% CHX statistically significant decreased the CFU counts. The difference of CFU counts for CHX0.2C were -1.31 log10 (p=0.009) and 1.26 log10 (p=0.001) for CHX0.2. (Figure 4A). An influence on biofilm mass was not found (Figure 4B) and the metabolic activity decreased only after the application of CHX0.5Cg (p<0.001) (Figure 4C).

Discussion

The present results have shown that the tested new CHX

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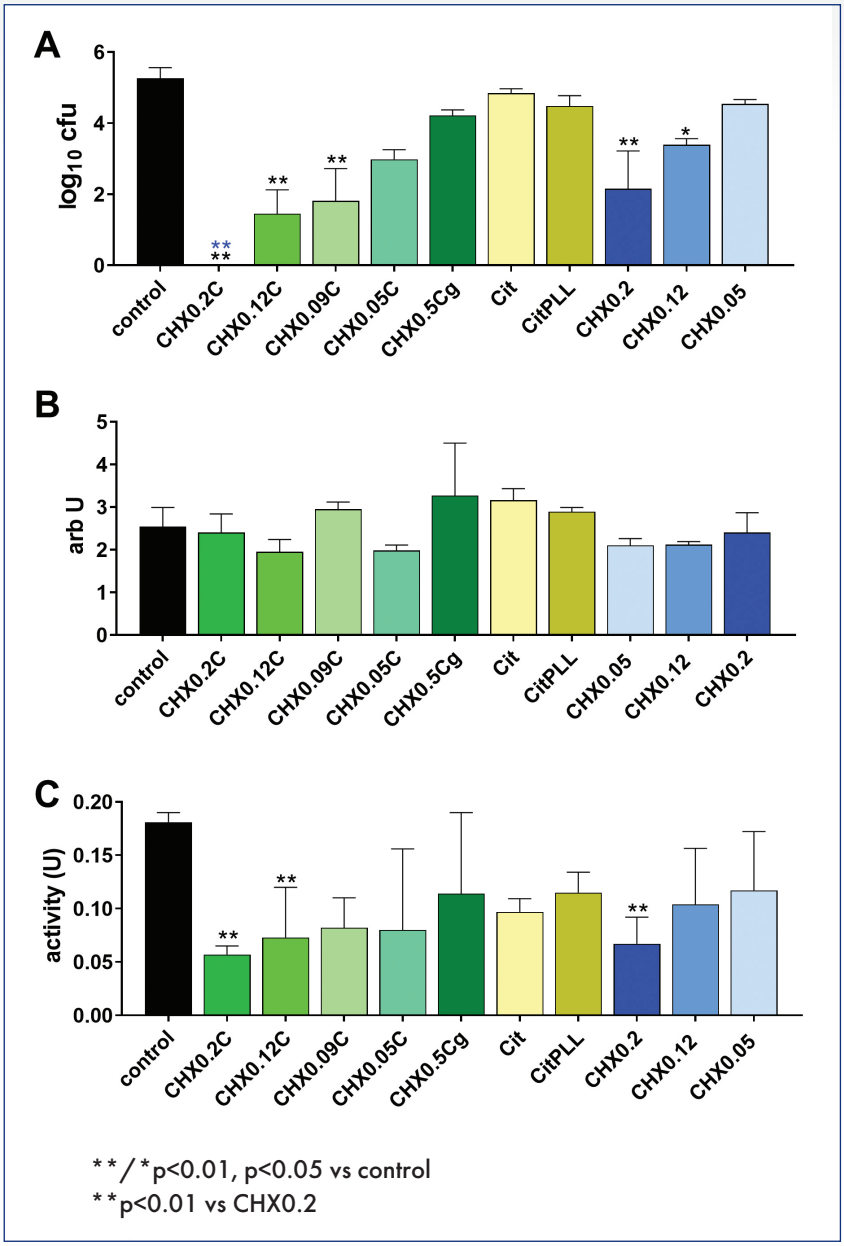

Figure 3: Activity of different formulations / controls on bacterial counts (A), mass (B) and metabolic activity (C) of the established "cariogenic" biofilm formed by five bacterial species for 48 h and after 1 h of exposition (1 min 100% of the formulation/solution, thereafter 10% for 1 h)
Tested formulations with CHX, Citrox® and poly-L-lysine: mouthrinsing formulations with 0.2% CHX (CHX0.2C), 0.12% CHX (CHX0.12C), 0.09% CHX (CHX0.09C) and 0.05% CHX (CHX0.05C) and a gel formulation with 0.5 CHX (CHX0.5Cg) Controls: 0.9% w/v NaCl as negative control (control), Citrox® preparations without (Cit) and with poly-L-lysine (CitPLL); CHX solutions without additives as positive controls with 0.2% CHX (CHX0.2), 0.12% CHX (CHX0.12) and 0.05 % CHX (CHX0.05).

formulations were active against the selected oral bacteria. They retarded biofilm formation to a greater extent than solutions with the same concentration of CHX without additives. The anti-biofilm activities depended on the CHX concentration within the formulations. However, as with the tested solutions, the formulations had only minor activity on an already formed biofilm.

In the present study, two different biofilm models and two different approaches were used. The biofilm models were designed to resemble caries and a periodontal disease. Defined strains were used to allow reproducible experiments with standardized conditions. One limitation of our study is the biofilm model used. The use of multispecies biofilms implies interaction between the various included species, but does not reflect the complexity present in the oral cavity, which consists of substantially more microorganism species. Using modern technologies, about 70 different microorganisms in caries[16] and about 300 in periodontal disease[17] were identified. Further limitations are the application and use of a static model. In the case of biofilm formation, the health-care formulations/solutions were applied only once and there was a constant concentration of 1% of the respective formulation/solution in the assay. In the established model, a 100% concentration of the formulations/solutions were

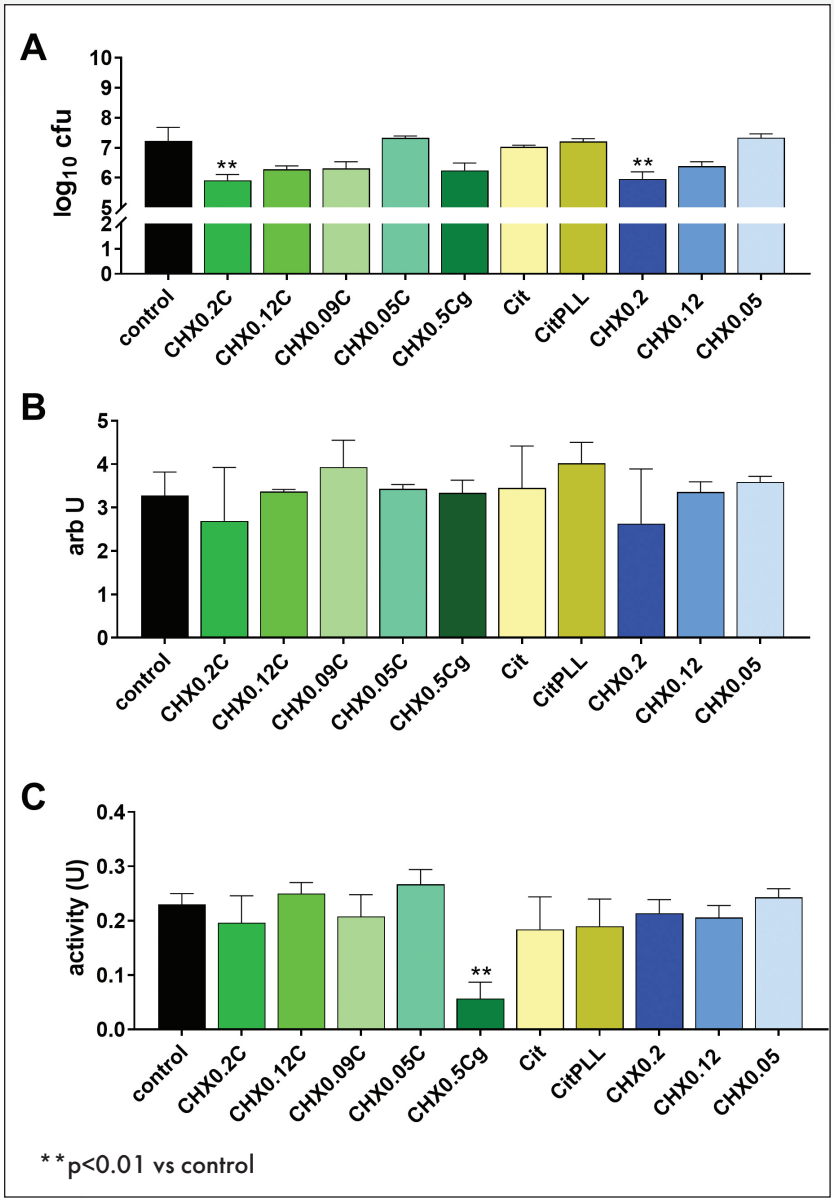

Figure 4: Activity of different formulations / controls on bacterial counts (A), mass (B) and metabolic activity (C) of the established "periodontal" biofilm formed by 12 bacterial species for 3.5 d and after 1 h of exposition (1 min 100% of the formulation/solution, thereafter 10% for 1 h)
Tested formulations with CHX, Citrox® and poly-L-lysine: mouthrinsing formulations with 0.2% CHX (CHX0.2C), 0.12% CHX (CHX0.12C), 0.09% CHX (CHX0.09C) and 0.05% CHX (CHX0.05C) and a gel formulation with 0.5 CHX (CHX0.5Cg)
Controls: 0.9% w/v NaCl as negative control (control), Citrox® preparations without (Cit) and with poly-L-lysine (CitPLL); CHX solutions without additives as positive controls with 0.2% CHX (CHX0.2), 0.12% CHX (CHX0.12) and 0.05 % CHX (CHX0.05).

applied for a short time before diluting to 10%. Limitations of the static biofilm are also visible in the 'cariogenic' biofilm model. When the different biofilms were formed, the log10 CFU counts of the cariogenic biofilm were higher after 6 h than after 24 h, whereas in the case of the periodontal biofilm, there was a continued increase. The 'cariogenic' biofilm consists mainly of streptococci, whereas anaerobically growing bacteria were dominant in the periodontal biofilm. The doubling time of streptococci is much quicker (4 – 6 h) compared with those of gram-negative anaerobes (20-24h),[18] suggesting that bacteria in the 'cariogenic' biofilm model consumed the available nutrients faster. Thus, the results obtained after 6 h of cariogenic biofilm formation might more closely resemble an in-vivo situation.

Citrox® was one of the additives in the tested formulations. It derives from citrus fruits, contains many different bioflavonoids and was first used as an additive in commercial sanitizer.[12] Citrox® is also in use as a food additive, where it is able to decrease the counts of certain pathogens such as *Salmonella* sp.[13] It has also been shown to be active against *Staphylococcus aureus* strains and to reduce the viability of biofilms.[19] Good to moderate activity was also found against oral microorganisms.[20] However, the results of the present study were different. MIC values were higher against oral streptococci, *Actinomyces* ssp., but lower against *P. gingivalis*, which may depend on the

cultivation media used. Furthermore, no activity by Citrox® on biofilm formation or an established biofilm was observed in our experiments. One explanation for this finding might be due to the fact that, in the present study, more-complex multi-species biofilm models were used.

Although no effect by Citrox® was found, the formulations were shown to inhibit biofilm formation. Even the low-concentration CHX formulations slowed 'cariogenic' biofilm formation more than higher-concentration CHX solutions without additives. This effect might be related to constituents other than Citrox®. All the formulations contained xylitol and poly-L-lysine. Xylitol has been described as an anti-adherent agent in biofilm formation.[21] In vitro, it inhibited formation of single-species biofilms of *S. mutans* and *S. sobrinus*[22] and also those of a dual-species biofilm by *S. gordonii* and *P. gingivalis*.[23] Poly-L-lysine has a strong antibacterial and anti-biofilm activity against *S. aureus*.[24] Functionalized titanium surfaces with poly-L-lysine containing silver nano-particles showed enhanced antimicrobial activity.[25] The effect was explained by the binding of poly-L-lysine to the negatively charged nanoparticles.[25] This cannot be assumed for binding to CHX as this is positively charged.[2] However, there might be a synergistic effect of binding to negatively charged surfaces as teeth and probably the plastic surfaces of microtiter plates.

As recently stated in a systematic review, despite the fact that CHX mouth rinses are able to reduce *S. mutans* counts in saliva, a definitive conclusion on its efficacy in preventing new caries lesions could not be drawn.[26] The efficacy of CHX mouth rinses on the reduction of *S. mutans* depends on their concentration[27] which was confirmed by our in-vitro study. Fluoride supplementation to CHX solution combines the fluoride retention in the oral cavity and the effects of CHX on the reduction of plaque, gingival inflammation and *S. mutans* counts.[28] In the present study, CHX0.05C containing sodium fluoride was in part more active than CHX0.05, which may support its use in preventing caries.

CHX0.09C was supplemented with hyaluronic acid. In dentistry, an adjunctive topical application may lead to additional clinical benefits in periodontal therapy.[29] Hyaluronic acid, a glycosaminoglycan is well known for its anti-inflammatory and wound-healing efficacy.[30] Hyaluronic acid inhibits bacterial adhesion and biofilm formation.[31] In the present study, CHX0.09C inhibited biofilm formation more than CHX0.12. Further research might be of interest to verify the role of hyaluronic acid as a component in mouth rinse solutions.

In the present in-vitro experiments, a gel formulation containing 0.5% CHX was also included. However, the results on bacteria counts were not superior to the use of 0.2% CHX solution. This is in agreement with findings of a systemic review that favored mouth rinse formulations to gels for clinical applications.[31]

Activitiy of CHX formulations/solutions was minor on an already formed 'periodontal' biofilm. Only the highest concentrations of 0.2% CHX used exerted some activity. This in vitro-result may once more underline the general guidelines that mechanical removal of a biofilm by scaling and root planing is essential in initial therapy of periodontitis.[33]

Conclusion

Taken together, the present in-vitro data support the anti-biofilm activity of the novel CHX, Citrox®, poly-L-lysine and xylitol oral health-care formulations. However, the biofilm inhibiting effect might not be related to Citrox® which cannot replace CHX-containing products. Further studies are warranted to confirm the present findings in various clinical settings.

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