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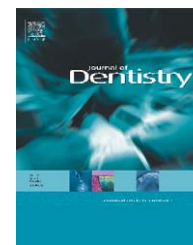
Efficacy of natural antimicrobials in toothpaste formulations against oral biofilms *in vitro*

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ABSTRACT

Objectives: To evaluate the antimicrobial efficacies of two toothpaste formulations containing natural antimicrobials (herbal extracts and chitosan) against oral biofilms of different composition and maturational status.

Methods: Bacteria from a buffer suspension or fresh saliva were adhered for 2 h to a salivary conditioning film and subsequently grown for 16 h. Dual-species biofilms were prepared from *Actinomyces naeslundii* T14V-J1 and *Streptococcus oralis* J22, whilst multi-species biofilms were grown from freshly collected human saliva. Biofilms were exposed to 25 wt% toothpaste supernatants. A chlorhexidine-containing mouthrinse and a buffer were used as positive- and negative-controls, respectively. Antibacterial efficacy was concluded from acute killing, bacterial removal, prevention of bacterial re-deposition and continued killing during re-deposition.

Results: The herbal- and chitosan-based supernatants showed immediate killing of oral biofilm bacteria, comparable with chlorhexidine. Moreover, exposure of a biofilm to these supernatants or chlorhexidine, yielded ongoing killing of biofilm bacteria after exposure during re-deposition of bacteria to a matured 16 h biofilm, but not to a much thinner initial biofilm formed by 2 h adhesion only. This suggests that thicker, more matured biofilms can absorb and release oral antimicrobials.

Conclusions: Supernatants based on herbal- and chitosan-based toothpastes have comparable immediate and ongoing antibacterial efficacies as chlorhexidine. Natural antimicrobials and chlorhexidine absorb in oral biofilms which contributes to their substantive action.

1. Introduction

Biofilm formation is a natural process in the oral environment, but needs to be controlled through regular brushing in order to prevent the development of caries and periodontal diseases. Regular toothpaste formulations contain a combination of fluorides and detergents, mainly sodium dodecyl sulphate to enhance the efficacy of brushing and thereby preventing diseases. Yet, in most people, brushing alone is inadequate to remove oral biofilm to an extent that the development of periodontal diseases and caries is prevented.[1] Therefore a variety of toothpaste and mouthrinse formulations with antibacterial properties have been developed and evaluated *in vitro* and *in vivo*. Common antimicrobials added are

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triclosan, stannous fluoride, cetyl pyridinium chloride and chlorhexidine.[2]

Despite the efficacy of many toothpaste formulations with antibacterial properties,[2,3] there is an increasing societal desire to rely on naturally occurring compounds for health care, which has also found its way into dentistry.[4] Parodontax®, for instance is a widely known herbal-based toothpaste, containing sodium bicarbonate and several herbal-containing components for which medicinal properties are claimed: chamomilla extract has anti-inflammatory properties, echinacea extract stimulates the immune response, salvia extract decreases tissue bleeding, myrrha extract is a natural antiseptic and the extract of mentha piperita is anti-septic, anti-inflammatory and antimicrobial.[5,6] Chitosan is another natural compound derived from the bio-polysaccharide chitin and has a poly-cationic carbohydrate structure. Chitin is the second most abundant biopolymer in nature and can be found in the exoskeletons of arthropods, shells of crustaceans and the cuticles of insects. Chitosan has many interesting properties amongst which non-toxicity and antimicrobial activity.[7] Applications of the antimicrobial activity of chitosans are currently investigated in food packaging, textile and cosmetic industries and in medicine, including dentistry.[8–10] Chitosan has antibacterial properties against oral bacterial strains[11–13] as well as the ability to adsorb to and change the physico-chemical properties of salivary conditioning films (or "pellicles"),[14] which suggests possible effects on bacterial re-deposition after use. Due to its cationic nature, however, it can be questioned whether these properties are preserved in a toothpaste formulation. Recently, however, the first toothpaste formulations containing chitosan have been made available on the market through the Internet (see, e.g. www.chitodent.de; www.dentachin.net).

The aim of this paper was to evaluate in vitro the antimicrobial efficacies of two toothpaste formulations containing natural antimicrobials (herbal extracts and chitosan) in terms of immediate and delayed bacterial killing in oral biofilms of different composition and maturational status. In addition, the antimicrobial efficacies of these natural antimicrobials were compared with the golden standard for chemical control of oral biofilms: chlorhexidine.

2. Materials and methods

2.1. Toothpastes supernatants, chlorhexidine and buffer

A herbal-based toothpaste, Parodontax® without fluoride (GlaxoSmithKline Consumer Healthcare B.V., Utrecht, The Netherlands) and Chitodent® (B&F Elektro GmbH, Filsum, Germany), a chitosan-based formulation were used. For biofilm exposure, a toothpaste supernatant was prepared by dissolving 25 wt% of toothpaste in adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride and 1 mM calcium dichloride, pH 6.8), which was centrifuged (10,000 × g, 5 min) to remove abrasive particles. Corsodyl®, a 0.2% chlorhexidine-containing mouthrinse (Corsodyl®, GlaxoSmithKline Consumer Healthcare B.V., Zeist, The Netherlands) and adhesion buffer were used as positive and negative controls, respectively.

2.2. Bacterial inocula

Actinomyces naeslundii T14V-J1 and Streptococcus oralis J22 were used for co-adhering dual-species biofilms. A. naeslundii was cultured in Schaedler's broth supplemented with 0.01 g/L hemin under anaerobic conditions and S. oralis in Todd Hewitt broth (THB, OXOID, Basingstoke, UK) in ambient air, both at 37 °C. Strains were precultured in an overnight batch culture and inoculated in a second culture which was grown for 16 h, harvested by centrifugation for 5 min at 6,500 × g and washed twice with adhesion buffer. To break bacterial chains or aggregates, bacteria were sonicated intermittently whilst cooling on ice for 30–40 s at 30 W. This procedure was found not to cause cell lysis. Bacteria were diluted to a cell density of 1×10^8 per mL for A. naeslundii and 3×10^8 per mL for S. oralis in adhesion buffer with 2% growth medium. The S. oralis suspension was supplemented with 1.5 mg/mL lyophilized human whole saliva.

Freshly collected human whole saliva from two healthy volunteers, with 7 filled teeth on average and stimulated by chewing Parafilm®, was used as a source for multi-species biofilms. In the morning, fresh saliva was collected and the bacterial density was determined by counting and found to amount $3 \pm 1 \times 10^8$ per mL on average. The two saliva samples were mixed and diluted 1:1 with adhesion buffer, therewith reducing the bacterial concentration to 1.5×10^8 per mL for initial adhesion. For growth, fresh human whole saliva from the same volunteers was centrifuged, in order to remove bacteria, tissue cells and debris, for 10 min at 10,000 × g at 10 °C. Subsequently, the saliva was filter sterilized by using a 1.2 μm filter followed by a 0.45 μm filter. Saliva was diluted to 10% in adhesion buffer in order to obtain a solution with a viscosity that can be used in the parallel plate flow chamber. All volunteers gave their informed consent to saliva donation, with approval of the Medical Ethical Committee at UMCG, Groningen (M09.069162), The Netherlands.

2.3. Biofilm formation and exposure to antimicrobials

First a salivary conditioning film was formed on microscope glass slides (75 mm × 25 mm). To this end, human whole saliva from at least 20 healthy volunteers of both genders was collected into ice-cooled beakers after stimulation by chewing Parafilm®. The saliva was pooled, centrifuged and treated by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM as a protease inhibitor in order to inhibit salivary protein denaturation. The solution was again centrifuged, dialyzed (molecular weight cut off, 6–8 kD) overnight at 4 °C against demineralized water, and lyophilized in order to effectively store saliva in unaltered form until needed.[15,16] For experiments, lyophilized saliva was dissolved at a concentration of 1.5 mg/mL in adhesion buffer. Glass slides were incubated in this reconstituted saliva for 16 h at room temperature.

Glass slides with a salivary conditioning film were used as the bottom plate of a parallel plate flow chamber (dimensions: $l \times w \times h$ = 175 mm × 17 mm × 0.75 mm, see Fig. 1).[17] The flow chamber was mounted on the stage of a phase contrast microscope equipped with a 40× ultra-long working distance objective (Olympus ULWD-CD Plan 40 PL). The flow chamber
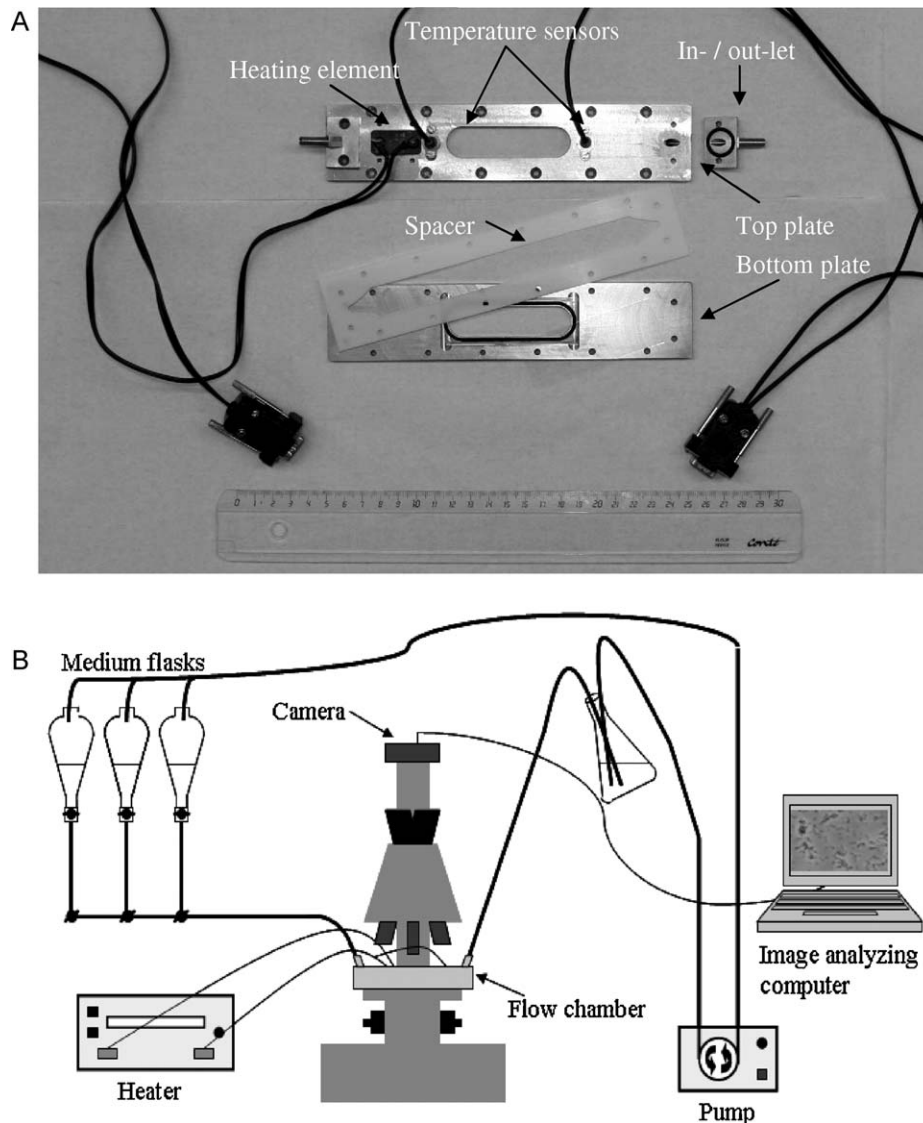
Fig. 1 – (A) Parallel plate flow chamber consisting of bottom plate, spacer, top plate and in-/out-let. The open space in the flow chamber is designed to hold a substratum (glass) plate. Temperature sensors and heating element are attached as well. (B) Basic design of the entire flow system used, shown with only one flow chamber connected.

and all tubing were sterilized before use. Prior to each experiment, all tubes and the flow chamber were filled with adhesion buffer and air bubbles were removed from the system. Once the system was filled, and prior to the addition of a bacterial suspension, adhesion buffer was pre-flowed for 30 min through the system in order to remove remnants of saliva and allow the system to warm up to 33 °C, a relevant oral surface temperature.[18] Solutions were circulated through the system at a constant flow rate of 1 mL/min, corresponding with a wall shear rate of $10\,s^{-1}$ which represents a moderate oral shear.[19]

The biofilms used in this study were dual-species biofilms of co-adhering bacteria and multi-species biofilms of oral bacteria from freshly collected saliva. These biofilms were prepared by initial 2 h adhesion and by initial adhesion followed by growth for 16 h. For dual-species biofilms, *A. naeslundii* was flowed until an arbitrary chosen surface coverage of 1×10^6 bacteria/cm^2 was reached. Subsequently,

flow was switched to buffer to remove unattached bacteria from the flow chamber and tubes for 30 min. Co-adhesion was initiated by switching the flow to *S. oralis* in saliva for 2 h, resulting in an initial biofilm. When appropriate, flow was switched to THB as a growth medium after initial adhesion and continued for 16 h at the same flow rate, followed by a 30 min buffer flow to remove THB and unattached bacteria. Initial biofilms of 2 h and biofilms after 16 h growth were exposed to toothpaste supernatants or controls for 10 min by perfusing the flow chamber and subsequent to 2 h re-deposition of *S. oralis*. After exposure to antimicrobials, the flow was switched to adhesion buffer for 30 min to remove unattached bacteria from the flow chamber and tubes.

For multi-species biofilms, initial bacterial adhesion from fresh human whole saliva was achieved by flowing with a 50% dilution of saliva for 2 h and continued, when appropriate, by a 16 h flow with a filter-sterilized 10% saliva solution as a growth medium at 0.5 mL/min, corresponding to a wall shear rate of

$5 s^{-1}$, to limit the volume of saliva required. After overnight growth, the antimicrobial exposure procedure described above, was performed. Three flow chambers were simultaneously operated, to allow biofilm evaluation before and after exposure, as well as after re-deposition of bacteria from saliva in one and the same experiment.

After growth, antimicrobial exposure and/or bacterial re-deposition, one of the three flow chambers was disconnected and biofilms were stained for 30 min *in situ* with live/dead stain (*Bac*Light[TM], Invitrogen, Breda, The Netherlands). Eight image stacks along the length of the flow chamber were taken using a Leica TCS-SP2 Confocal Laser Scanning Microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany), whilst scanning from bottom to top of the biofilm. Images were analysed with COMSTAT,[20] a Matlab® (The Mathworks, Inc.) based analysis program. COMSTAT enables measurement of the biofilm volume ($\mu m^3/\mu m^2$) occupied by live and dead bacteria, from which the %live bacteria in a biofilm volume and the %removal after flowing with toothpaste supernatant or chlorhexidine (positive control) or buffer (negative control) is obtained according to

$$\%removal = \left(\frac{biofilmvolume_{before} - biofilmvolume_{after}}{biofilmvolume_{before}}\right) \times 100\% \qquad (1)$$

in which biofilmvolume$_{before}$ and biofilmvolume$_{after}$ represent the total biofilm volumes before and after exposure to a toothpaste supernatant or control solutions.

2.4. Statistical analysis

Statistical analysis and comparison of the different groups were performed with Student's paired samples t-test for comparison before and after exposure to buffer or antimicrobial agent and Student's independent samples t-test for comparisons between the different biofilm models. A significance level of $p < 0.05$ was used.

3. Results

3.1. Initial biofilms

Total biofilm volume of the initial dual-species biofilm after 2 h adhesion amounted on average $2.4 \pm 1.0 \ \mu m^3/\mu m^2$ and these biofilms possessed a high (95%) viability (see Fig. 2A). Biofilm volumes after 2 h initial adhesion of bacteria from whole saliva (multi-species biofilms) were only around $0.2 \ \mu m^3/\mu m^2$ and therewith too small for further analyses.

Chlorhexidine and herbal- and chitosan-based toothpaste supernatants removed significantly ($p < 0.05$) more bacteria than buffer (see Fig. 3), with no significant differences in removal between chlorhexidine and both toothpaste supernatants. The viabilities of 2 h old, initial biofilms were significantly reduced after exposure to toothpaste supernatants and chlorhexidine, but most pronounced for chlorhexidine (see also Fig. 2A).
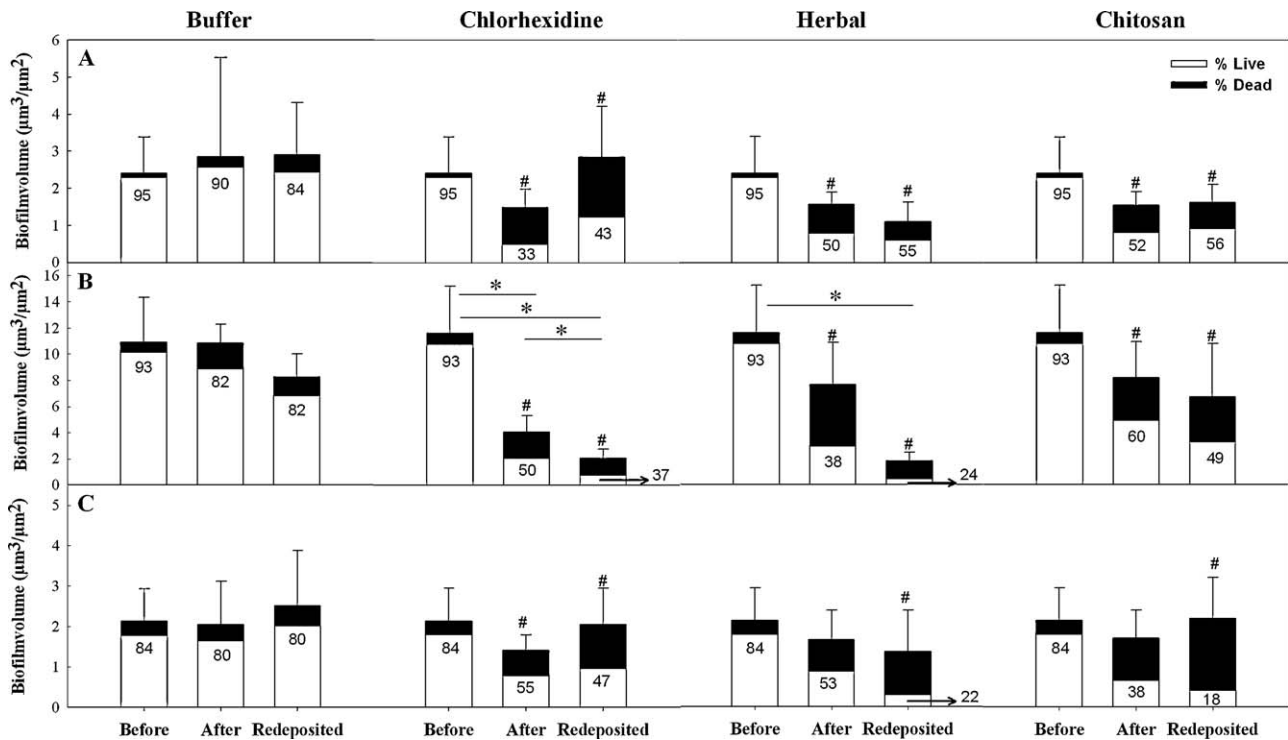

Fig. 2 – The total biofilm volume ($\mu m^3/\mu m^2$) of oral biofilms on saliva-coated glass, including the %live (also indicated in numbers) and %dead bacteria before and after exposure, as well as after 2 h re-deposition: (A) 2 h old, initial dual-species biofilms, (B) 16 h old dual-species biofilms, and (C) 16 h old multi-species biofilms. Error bars represent the SD in total biofilm volume over three experiments with separately cultured bacteria. #Significantly ($p < 0.05$) lower viability than before treatment. *Significantly ($p < 0.05$) difference in biofilm volume.
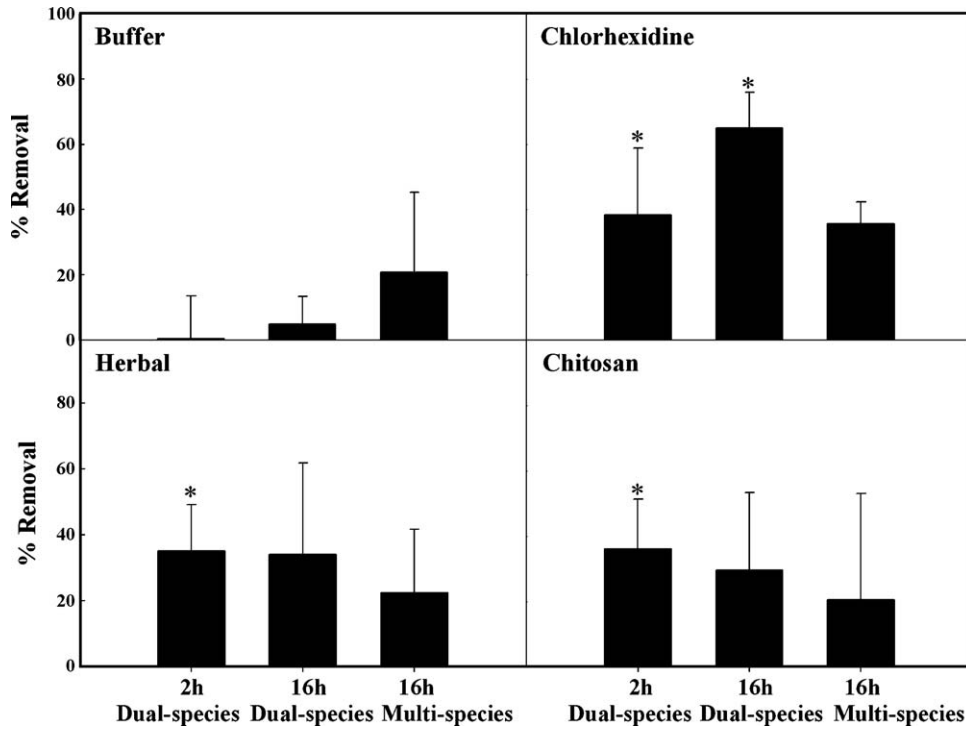
Fig. 3 – %Removal of 2 h old, initial biofilms, 16 h old dual-species biofilms and 16 h old multi-species biofilms by exposure to buffer, chlorhexidine and supernatants of a herbal- or chitosan-based toothpaste formulations. Error bars represent the SD over three experiments with separately cultured bacteria.
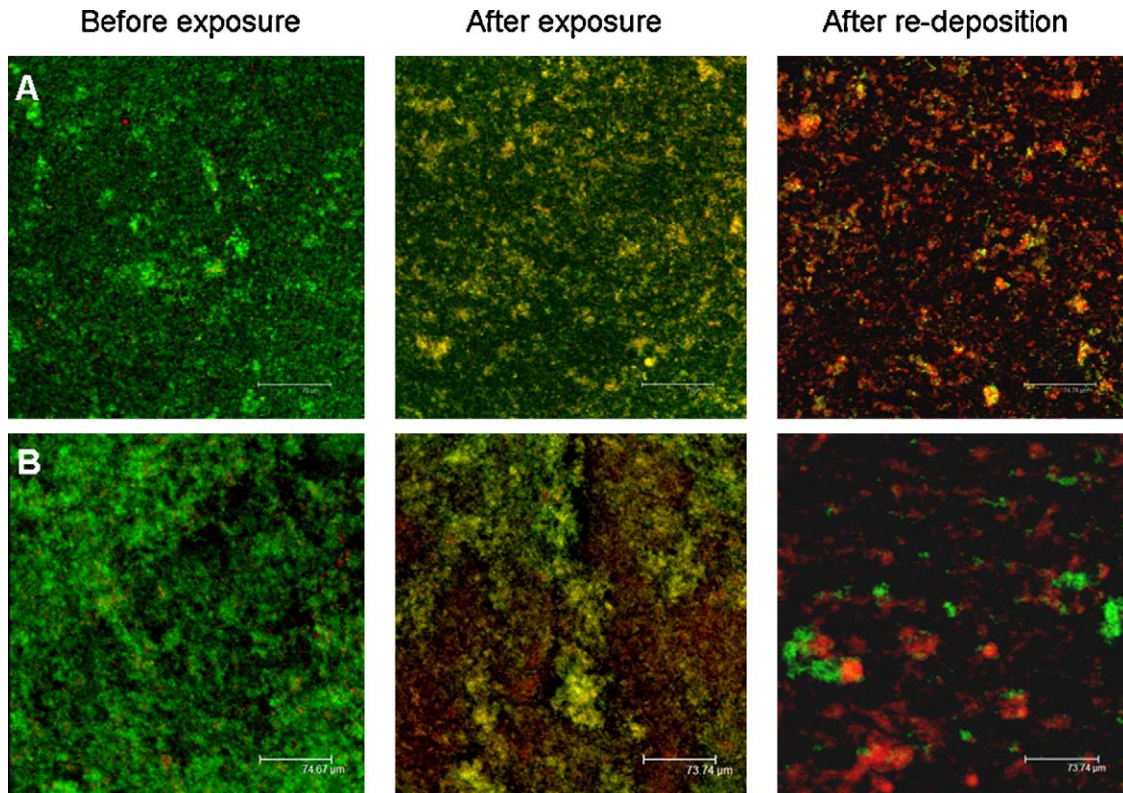
Fig. 4 – Confocal laser scanning microscopic overlay images of 16 h old dual-species biofilms before and after exposure to herbal-based toothpaste supernatant (A) or chlorhexidine (B), as well as after 2 h re-deposition of bacteria. Staining was done with live/dead stain: green represents live bacteria whilst dead bacteria appear as red fluorescent dots. Bar denotes 75 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Re-deposition of *S. oralis* to treated 2 h old, initial biofilms was evident from an increased biofilm volume after exposure to chlorhexidine, although not statistically significant. No adhesion of *S. oralis* during the re-deposition phase was observed on biofilms exposed to herbal- and chitosan-based toothpaste supernatants, but moreover ongoing removal was seen in the case of the herbal-based toothpaste (Fig. 2A).

3.2. Mature biofilms

Total biofilm volume of the dual-species biofilm after 16 h formation amounted $10.9 \pm 3.4\ \mu m^3/\mu m^2$, which is about four-fold thicker than after 2 h growth. Multi-species biofilms grown from saliva had a significantly smaller ($p < 0.05$) biofilm volume ($2.1 \pm 0.8\ \mu m^3/\mu m^2$) than dual-species biofilms.

All biofilms after 16 h growth (see Fig. 2B and C) were highly viable before exposure to the natural antimicrobials and chlorhexidine, although the 16 h old dual-species biofilm was slightly more viable (93%) than the multi-species one (84%). Chlorhexidine was significantly more effective in removing dual-species biofilm compared with buffer (65%, see Fig. 3) than the two toothpaste supernatants. Exposure to the herbal- or chitosan-based toothpaste supernatant removed 21–34% of the biofilm volumes, respectively (see Fig. 3), concurrent with a significant decrease in viability for both formulations to less than 60% (see Fig. 2). Chlorhexidine detached more bacteria on a percentage basis from the 16 h old biofilm than from the 2 h old biofilm. The decrease in viability achieved by chlorhexidine was comparable or less than to the ones of the herbal- or chitosan-based toothpaste supernatants. Buffer exposure neither caused biofilm removal nor a decrease in viability (see Figs. 2 and 3).

Adhesion of bacteria during the re-deposition phase was only observed after exposure to chlorhexidine and the chitosan-based toothpaste supernatant of a multi-species biofilm (Fig. 2C), indicated by an increase in biofilm volume, whereas the herbal-based toothpaste supernatant showed a slight ongoing removal. For dual-species biofilms (Fig. 2B), all antimicrobial agents caused ongoing removal even during the re-deposition phase of the experiment, which was statistically significant for chlorhexidine. Moreover, during the re-deposition phase, bacterial viabilities continued to decrease to below the levels observed prior to the bacterial re-deposition phase. This is also illustrated in the CLSM micrographs of 16 h old dual-species biofilms before and after exposure to herbal-based toothpaste supernatant and chlorhexidine (see Fig. 4). Note that bacteria appear yellowish immediately after exposure due to the superposition of red and green-fluorescent bacteria present over the thickness of the biofilm. After re-deposition, however, bacteria appear more convincingly as red, indicative of ongoing killing during the re-deposition phase.

4. Discussion

In this paper, we studied antibacterial efficacies of two toothpastes containing natural antimicrobial components in comparison with the efficacy of chlorhexidine, being the gold standard for chemical oral biofilm (or ''plaque'') control.

Antibacterial efficacy was assessed against biofilms of different maturational status, grown for 2 or 16 h and comprised of *A. naeslundii* and *S. oralis*, two initial colonizers of enamel surfaces *in vivo*, including multi-species biofilms grown for 16 h from saliva.

Exposure of biofilms to both the herbal- or the chitosan-based toothpaste derived supernatants yielded comparable or better immediate and ongoing killing than chlorhexidine exposure and even prevention of bacterial adhesion during the re-deposition phase to the biofilm was seen. This could only be demonstrated in mature biofilms, formed by growth during 16 h, therewith suggesting a larger absorptive capacity of thicker biofilms, that may be involved in the substantive action of the antimicrobials. Clinical studies have also shown the potential of both Parodontax and chitosan in reducing plaque re-growth as well as antibacterial substantivity.[21,22]

Chlorhexidine is known to possess good substantivity that may enhance its clinical efficacy when long-term killing is needed.[23] Interestingly, the present study also showed that chlorhexidine, due to its surfactive properties,[24] detached, more bacteria on a percentage basis from a mature biofilm than from an initial biofilm, which might indicate that the adhesion strength between a bacterium and the salivary conditioning film is stronger than between bacteria in the biofilm. It is known that chlorhexidine influences the composition of the oral biofilms, and especially the prevalence of actinomyces decreased after use of chlorhexidine.[25] Our results show that the percentage live bacteria in our dual-species biofilms is lower than in multi-species biofilms, which might be explained by a greater killing efficacy of chlorhexidine with respect to actinomyces. Chlorhexidine and chitosan have the same mechanism of antimicrobial activity, as both disrupt the bacterial cell membrane, leading to cell death.[26,27] The mechanisms of antimicrobial activities of the components in Parodontax are unclear.

Adhesion of bacteria during the re-deposition phase to the biofilms was only seen after exposure to chlorhexidine and chitosan-based toothpaste supernatants in a multi-species biofilm. Likely, the variety of different bacterial strains and species in saliva offers the possibility for different bacterial strains to adhere to an exposed biofilm, whereas the two strains constituting the dual-species biofilm are not attracted to these biofilms. This highlights the major advantage, and at the same time the disadvantage of using multi-species biofilms grown from saliva. Due to the large variety of strains and species, the experiment becomes less defined than when working with a dual-species biofilm, but on the other hand, multi-species biofilm are a better representation of biofilms as clinically occurring. In addition, increased re-deposition of bacteria after exposure to these two cationic antimicrobials may be stimulated by their adsorption to the salivary conditioning film, which is known to be accompanied by less negative zeta potentials and thus less electrostatic repulsion between negatively charged bacteria and the conditioning film.[14]

It can be of clinical relevance that bacterial detachment and killing in mature oral biofilms may continue after exposure to antibacterial compounds from toothpastes and chlorhexidine. This suggests that matured biofilms may act as a reservoir for oral antimicrobials enabling prolonged killing, whereas initial

biofilms are evidently too thin to act as an effective reservoir. These observations confirm recent clinical findings by Otten et al.,[28] demonstrating that clinically collected plaques from patients after rinsing with an antibacterial mouthrinse indeed contained sufficient antibacterial activity to kill new plaque.

We here demonstrated *in vitro* that natural antimicrobials in herbal- and chitosan-based toothpastes can be equally effective as chlorhexidine, not only with respect to immediate but also delayed bacterial killing as a result of substantivity of antimicrobials in oral biofilms.

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