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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