A dentifrice containing salivary enzymes and xylitol exhibits superior antimicrobial activity *in vitro* against oral biofilms containing *Streptococcus mutans* compared to a chlorhexidine dentifrice

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Significance and impact

Antimicrobial oral hygiene products have an important role in controlling oral diseases such as dental caries and periodontal disease. Chlorhexidine is the gold standard antimicrobial in such products but is often associated with staining. In order to find improved antimicrobials with greater antiplaque activity, we investigated a combined salivary enzyme complex (SEC)/xylitol dentifrice for antimicrobial activity. Our SEC/xylitol formulation shows enhanced anti-biofilm activity compared to chlorhexidine dentifrice, including higher activity against biofilms of the cariogenic bacterium *Streptococcus mutans*. SEC/xylitol combination dentifrices show promise as natural antimicrobial alternatives to chemical antimicrobials for the control of oral diseases.

Abstract

Human saliva contains natural antimicrobial enzymes. In this in vitro study, we evaluate the antimicrobial activity of a dentifrice containing a salivary enzyme complex (SEC) with xylitol versus a standard 0.12% chlorhexidine (CHX) dentifrice. Biofilms of *Streptococcus gordonii*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Fusobacterium nucleatum* subsp *polymorphum* and *Corynebacterium matruchotii* were exposed to SEC and CHX dentifrices for 2 mins and viable CFUs were enumerated. Exposure to the SEC dentifrice resulted in a significant reduction in biofilm viability, which was greater than that shown by the CHX dentifrice, against all organisms tested. The SEC dentifrice also exhibited greater antimicrobial activity against all organsims in well diffusion assays compared to CHX. Dentifrice activity was also evaluated against a three species biofilm of *Streptococcus gordonii*, *Streptococcus mutans* and *Corynebacterium matruchotii* using bacterial live/dead stain. The SEC dentifrice was at least as effective as CHX in removal of the multispecies biofilm. The combination of SEC and xylitol generates a highly effective antimicrobial dentifrice with greater anti-biofilm activity than a standard 0.12% CHX formulations. SEC and xylitol combinations are worthy of further investigation for routine use and in the management of gingivitis and periodontal disease.

Key words: Dentifrice, chlorhexidine, enzyme, biofilm, antimicrobial, *Streptococcus mutans*

Introduction

Many mammalian secretions, including milk, saliva and respiratory mucous, contain active antimicrobial proteins that exhibit antibacterial, antifungal or antiviral activity (Vasstrand and Jensen 1984; Tenovuo 2002; Cawley *et al.* 2019; Magacz *et al.* 2019; Nakano, Tanaka and Abe 2020). These antimicrobial proteins include lysozyme, lactoferrin, lactoperoxidase and a variety of small antimicrobial peptides (Vasstrand and Jensen 1984; Roger *et al.* 1994; Pinheiro *et al.* 2020). These enzymes and proteins have varied mechanisms of action including hydrolysis of bacterial cell walls (lysozyme), iron sequestration (lactoferrin) and oxidative attack on microbial cell surfaces (lactoperoxidase). Lactoperoxidase (LPO) catalyses the oxidation of many inorganic substrates by H_2O_2 to generate reactive oxygenated derivatives. The most significant of these substrates in the oral cavity is thiocyanate (SCN^-) which is oxidised to form hypothiocyanite ($OSCN^-$). LPO in saliva has significant antimicrobial activity largely mediated through the production of hypothiocyanite ($OSCN^-$) ions which are thought to oxidise microbial surface proteins and exhibit a microbicidal effect (Bafort *et al.* 2014). This reaction involves the initial oxidative activation of the native LPO enzyme by H_2O_2 in saliva to form compound I (Magacz *et al.* 2019). The active compound I can then carry out the oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$). Thiocyanate ions are the preferred substrate of the active enzyme and these are naturally found in saliva. The oxidised hypothiocyanate is highly reactive and can react with thiol groups on bacterial proteins and this has a bactericidal effect (Thomas and Aune 1978). In saliva, LPO works in synergy with other enzymes including lactoferrin and lysozyme to regulate the oral microbiome and prevent oral disease. LPO has been shown to have activity against planktonic and biofilm growing oral bacteria and may inhibit bacterial biofilm formation on tooth surfaces due to its ability to adhere to the salivary pellicle (Roger *et al.* 1994).

There now exists an extensive literature showing the effectiveness of dentifrices containing natural enzymes, including LPO, in terms of antimicrobial activity and oral healthy promoting properties (reviewed by Magacz *et al.* (Magacz *et al.* 2019)). In vitro, antimicrobial activity of LPO has been demonstrated against cariogenic *S. mutans* and also Gram negative periodontal pathogens such as *P. gingivalis* and also multispecies biofilms (Roger *et al.* 1994; Welk *et al.* 2009; Cawley *et al.* 2019). In human trials LPO dentifrices have been shown to reduce plaque scores, reduce gingival bleeding and remission of symptoms of dry mouth (Kirstilä *et al.* 1996; Epstein 1999; Tenovuo 2002; Jyoti, Shashikiran and Reddy 2009; Nakano *et al.* 2019; Pinheiro *et al.* 2020; Welk *et al.* 2021). In human trials, LPO has been associated with increased levels of hypothiocyanate (Lenander-Lumikari, Tenovuo and Mikola 1993) and reduced levels of *S. mutans* and periodontal pathogens such as *P. gingivalis* and *F. nucleatum* (Jyoti, Shashikiran and Reddy 2009; Nakano, Tanaka and Abe 2020; Rabe *et al.* 2022).

Xylitol has also been incorporated in dentifrices and has been proposed to have several antibacterial mechanisms of action, including disruption of bacterial energy metabolism and direct antimicrobial activity (Benahmed *et al.* 2020; Teng, Xixian and Ismail 2022). Interestingly, some studies have indicated that xylitol may enhance LPO activity in the oral cavity and in vitro (Mäkinen, Tenovuo and Scheinin 1976; Kim *et al.* 2015). Mäkinen et al. provided evidence that ingestion of xylitol increased LPO activity in vivo in volunteers who ingested xylitol sweeteners (Mäkinen, Tenovuo and Scheinin 1976). Similarly, in vitro studies by Kim et al. showed that xylitol enhanced the enzymatic activity of salivary LPO (Kim *et al.* 2015) .

Activity of LPO against oral biofilms has been demonstrated with in vivo and in vitro studies (Modesto, Lima and Uzeda 2000; Rabe *et al.* 2022). Attempts to replicate dental biofilm growth in the laboratory often involves growth of multiple bacterial species on solid surfaces coated with saliva (Paqué *et al.* 2022). In the current study, we use in vitro grown biofilms to

examine the antibacterial and antibiofilm activity of a dentifrice containing a salivary enzyme complex (SEC) combined with xylitol. As a control, we use the gold-standard antimicrobial agent, chlorhexidine. We examine activity against biofilms of a variety of common pathobionts including *S. mutans*. We also examine activity against organisms known to be important for plaque maturation and development, including *F. nucleatum*, which acts as a bridge species to allow incorporation of many Gram negative periodontal pathogens into plaque biofilms (Zijnge *et al.* 2010). In addition, we examine activity against *Corynebacterium matruchotii*. Recent studies of plaque architecture on human teeth have shown that *Corynebacterium* species play an important role as a scaffold for other bacteria to bind to in plaque biofilms (Welch *et al.* 2016). We examine single species *Corynebacterium matruchotii* biofilms and a simple multispecies biofilm incorporating *S. gordonii*, *S. mutans* and *Corynebacterium matruchotii*.

Materials and Methods

Bacterial strains and culture conditions.

Bacterial strains were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures GmbH or the UK National Collection of Type Cultures (NCTC). These included *Streptococcus gordonii* DL-1, *Streptococcus mutans* NCTC10449, *Actinomyces naeslundii* DSM43013, *Fusobacterium nucleatum* subsp *polymorphum* NCTC10953 and *Corynebacterium matruchotii* DSM20635.

F. nucleatum and *A. naeslundii* were cultured anaerobically in Brain Heart Infusion (BHI) broth in 2.5 l anaerobic jars (Oxoid) using the AnaeroGen gas generating system (Oxoid).

All other bacteria were cultured aerobically at 37˚C in BHI broth in 250 ml Erlenmyer flasks with shaking at 250 rpm. Aerobic and anaerobic plate culture was carried out with BHI agar at 37˚C.

Dentifrices

Two dentifrice preparations were compared. The base formulations of both preparations were identical. One contained chlorhexidine (0.12% w/v) as the active antimicrobial ingredient. The second preparation consisted of a lactoperoxidase containing salivary enzyme complex (SEC) supplemented with xylitol. Samples of both preparations are available by request from the authors. Dentifrices were tested as 30% v/v suspensions prepared by vigorous mixing with Dulbecco's modified Eagle's medium (DMEM).

Biofilm assay

Biofilms were grown on the surface of plastic 12-well dishes (Greiner Bio-one). The surface of the dish was pre-treated with human saliva for 24 h prior to the assay. Saliva was unstimulated and was recovered from healthy adult volunteers by sampling in a sterile 50 ml tube. Collected samples were pooled and centrifuged at 4,000 x g for 20 min at 4˚C. Samples were then UV sterilised for 30 minutes and aliquoted in 1.5 ml Eppendorf tubes. Sterility was checked prior to use by direct aerobic culture. Samples were stored at -80˚C.

Bacteria were grown in BHI broth to the late logarithmic phase of growth. Bacteria collected by centrifugation at 10,000 x *g* and washed twice in Dulbecco's Modified Eagles Medium (DMEM, Gibco). The OD600nm of the bacterial suspension was measured and the density was adjusted yield a suspension of ~1 x 10^7 bacteria/ml. A 500 µl aliquot of this suspension was added to 9 individual saliva coated wells and allowed to adhere for 2 h in a humid incubator set at 37˚C with 5% CO_2. Following incubation, the liquid was removed and the adherent biofilm was washed with 500 µl of fresh DMEM. Three wells supplemented with 500 µl of fresh DMEM and acted as controls, three wells were supplemented with 500 µl of a 30% v/v suspension of the chlorhexidine dentifrice and 3 wells were supplemented with a 500 µl volume of the SEC dentifrice. Biofilms were exposed for 2 mins or 10 mins. Following this incubation,

the antimicrobial or control suspension was removed and the biofilm was washed with 500 µl of PBS. A 1 ml volume of PBS was then added to each well and the remaining biofilm was removed by vigorous pipetting. Each sample was vortexed and serially diluted 10-fold and triplicate plate counts were performed to assess bacterial viability on BHI agar. Data were analysed and plotted using Prism GraphPad (San Diego, California, USA). Data were analysed using a Kruskal-Wallis tests with Dunn's test for pairwise comparisons.

Well diffusion assay

Bacterial suspensions were prepared in DMEM as described for biofilm assays and were diluted 1/10 in DMEM. A BHI agar plate containing 25 ml agar was prepared for each assay by punching a 10 mm diameter hole in the agar. Using a cotton swab, the bacterial suspension was spread evenly over the surface of the plate and allowed to dry. A 100 µl aliquot of DMEM or dentifrice suspension (30% v/v in DMEM) was then added to the well and the place was incubated at 37˚C aerobically (or anaerobically for *F. nucleatum* and *A. naeslundii*) until sufficient growth was achieved to discern a halo (24-48 h). Halo size was recorded using a Flash n' Go plate visualizer (IUL Instruments) and each experiment was done triplicate.

Visualisation of biofilm removal

In order to visualise biofilm removal and bacterial killing, a qualitative assay was carried out using the LIVE/DEAD Baclight bacterial viability kit (Thermofisher). A suspension of (2 x 10^7) of each of *S. gordonii*, *S. mutans* and *C. matruchotii* was used to generate a multispecies biofilm in the same fashion as described above. The biofilm was exposed to DMEM (control) or to a 30% v/v suspension of dentifrice for 2 minutes. The suspension was removed and the biofilm was washed (3 x) with 1 ml of PBS. The biofilm was then stained with the LIVE/DEAD Baclight stain and visualised using a Zoe inverted fluorescent microscope (BioRad).

Results and Discussion

Biofilm viability

S. gordonii biofilms formed on saliva coated plastic wells were exposed to either a chlorhexidine dentifrice (CHX) or a salivary enzyme complex dentifrice (SEC) for 2 or 10 minutes (Figure 1). Viable counts showed an approximate 3 log reduction in CFUs following 2 min exposure to the SEC formulation which was highly significant (P=0.0073) compared to the CHX treatment (P=0.18). Following a 10 minute exposure to SEC we observed a greater reduction in viability of the *S. gordonii* biofilm (P=0.007). Additional assays were carried out to compare the antimicrobial effects on other plaque forming organisms including *F. nucleatum*, *C. matruchotii*, *S. mutans* and *A. naeslundii* (Figure 1). In the case of the Gram-positive organisms, an approximate 3 log reduction in viability was observed after 2 min exposure to SEC which was significant (all $P < 0.05$). In the case of the Gram negative organism *F. nucleatum* we observed a ~2-log reduction in viability (P=0.015). In each case the effective drop in viable CFUs was significantly greater with the SEC formulation compared to the CHX dentifrice. In the case of *F. nucleatum*, *C. matruchotii*, *S. mutans* and *A. naeslundii*, a 10 min exposure yielded similar results to the 2 minute exposure (data not show).

Well diffusion assays

We examined the capacity of CHX and SEC dentifrices (30% v/v) to inhibit the growth of agar adherent biofilms in well diffusion assays (Figure 2). In this assay format, each organism tested yielded a larger halo of inhibition with the SEC dentifrice compared to the CHX formulation. *S. mutans* appeared to exhibit the least susceptibility to SEC, however the activity of SEC against *S. mutans* was reproducibly greater than the CHX formulation. *F. nucleatum* exhibited the greatest susceptibility to SEC in this format.

Biofilm visualisation

A qualitative assessment of multispecies biofilm viability and removal was carried out using the LIVE/DEAD Baclight bacterial viability kit (Thermofisher). A tri-species biofilm of *S. gordonii*, *S. mutans* and *C. matruchotii* was grown and exposed to DMEM (control) or to a 30% v/v suspension of each dentifrice for 2 minutes. The LIVE/DEAD Baclight stain allowed visualisation of viable (green) and dead (red) fluorescing bacteria (Figure 3). Without treatment we could observe microcolonies of bacteria which exhibited green fluorescence only, indicating high levels of viability. Treatment with CHX dentifrice (30% v/v) for 2 mins resulted in decreased levels of adherent biofilm and an increase in red fluorescence indicating loss of bacterial viability. Treatment with SEC dentifrice (30% v/v) also resulted in increased red fluorescence and removal of biofilm at a level comparable to the CHX treatment. These observations were consistent in replicate experiments.

Conclusions

In conclusion, our study shows that a novel SEC dentifrice formulation exhibits greater antimicrobial activity in comparison to the gold-standard antimicrobial chlorhexidine. Chlorhexidine is used in many commercially available dentifrices used to treat gingivitis and periodontal disease (Brookes *et al.* 2021). However use of chlorhexidine is commonly associated with staining of teeth and prostheses and in rare cases it can cause irritation or allergic responses (Pałka, Nowakowska-Toporowska and Dalewski 2022). As antimicrobials, salivary enzymes offers some advantages over chemical biocides. As they are naturally occurring proteins, they exhibit excellent biocompatibility (Magacz *et al.* 2019). In addition, as microbes are naturally exposed to salivary enzymes *in vivo*, they should not result in increased selection of organisms resistant to clinically used antibiotics. LPO containing dentifrices have been shown to have good antimicrobial activity against biofilms of oral

250 microorganisms (Modesto, Lima and Uzeda 2000; Jones *et al.* 2018; Rabe *et al.* 2022). Clinical
251 trials have also shown that regular use of LPO containing dentifrices can reduced plaque levels
252 and improve gingival health (Nakano *et al.* 2019; Nakano, Tanaka and Abe 2020).
253 In the current study, we directly compare the antibiofilm activity of a novel xylitol/SEC
254 combination versus a standard 0.12% chlorhexidine dentifrice. In order to maximise SEC
255 activity, xylitol was included in the formulation. Although not extensively investigated, there
256 is some evidence that LPO activity is enhanced in the presence of xylitol, however the exact
257 mechanism for this has not been elucidated (Mäkinen, Tenovuo and Scheinin 1976; Kim *et al.*
258 2015). We initiated our investigations against biofilms composed of organisms considered to
259 be early colonisers of human teeth, namely *S. gordonii* and *A. naeslundii*. A 2 min exposure to
260 30% v/v SEC dentifrice was sufficient to cause a ~3 log reduction in viability compared to
261 controls, which was statistically significant compared to the effects of a chlorhexidine
262 dentifrice. Superior activity was also demonstrated against the Gram negative anaerobe *F.*
263 *nucleatum* and the Gram positive organism *C. matruchtii*. Both of these species were selected
264 for investigation due to their important role in plaque maturation (Zijnge *et al.* 2010; Welch *et*
265 *al.* 2016). *Corynebacterium* species have been shown to act as scaffold in supragingival plaque
266 and *F. nucleatum* has been shown to act as bridge between supragingival and subgingival
267 plaque, allowing biofilm incorporation of late colonisers such as *P. gingivalis* (Kolenbrander
268 and Andersen 2006). The activity against these species supports a mechanism whereby SEC
269 can disrupt plaque maturation. We also observed a significant 3-log reduction in the viability
270 of *S. mutans*, an organism with a major role in the development of dental caries, suggesting a
271 caries protective role. This is in agreement with numerous studies that have shown activity of
272 LPO against *S. mutans* (Roger *et al.* 1994; Modesto, Lima and Uzeda 2000; Jyoti, Shashikiran
273 and Reddy 2009; Welk *et al.* 2009).

These data were supported by well diffusion assays which also demonstrated the enhanced activity of SEC dentifrice compared to chlorhexidine formulations. *S. mutans* and *A. naeslundii* had the lowest susceptibility to chlorhexidine in this assay format with average zones of inhibition of 13 and 12 mm diameter, respectively. The SEC dentifrice showed enhanced activity against both species, almost doubling the size of the zone of inhibition in the case of *S. mutans*.

We also examined a combination of organisms in a mixed species biofilm, namely *S. gordonii*, *C. matruchotii* and *S. mutans.* Although this analysis was qualitative in nature, we observed that SEC was at least as effective as chlorhexidine formulations in removal of the multispecies biofilm and in reducing bacterial viability, as indicated by the level of red fluorescence.

Although our study shows excellent antimicrobial activity by the SEC dentifrice, we have not specifically addressed if the incorporation of xylitol enhances the activity of the enzyme complex, as suggested by some previous studies. Future studies comparing the SEC dentifrice with and without the xylitol addition will be required to address this.

Overall, our data indicate that an SEC dentifrice formulation can exhibit antimicrobial activity greater than chlorhexidine formulations. This activity supports a role for SEC formulations as excellent choices for individuals at high risk of caries or periodontal disease, or those with reduced manual dexterity who require extra antimicrobial support. Further research is required to determine the mechanistic nature of this antimicrobial combination.

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Conflicts of interests statement

DL is the Director of LA Research labs (manufacturer of oral healthcare products). The other authors have no conflicts of interest to declare.

Data Availability Statement

All data and materials are available on request from the authors.

Authors contribution statement

MO'C and GH were involved in assay design, carried out experimental assays, analysed the data and assisted with manuscript preparation. DL was involved in study conception and design, manuscript preparation and funding proposals. GPM was involved in study design, supervision of researchers, data analysis and manuscript writing.

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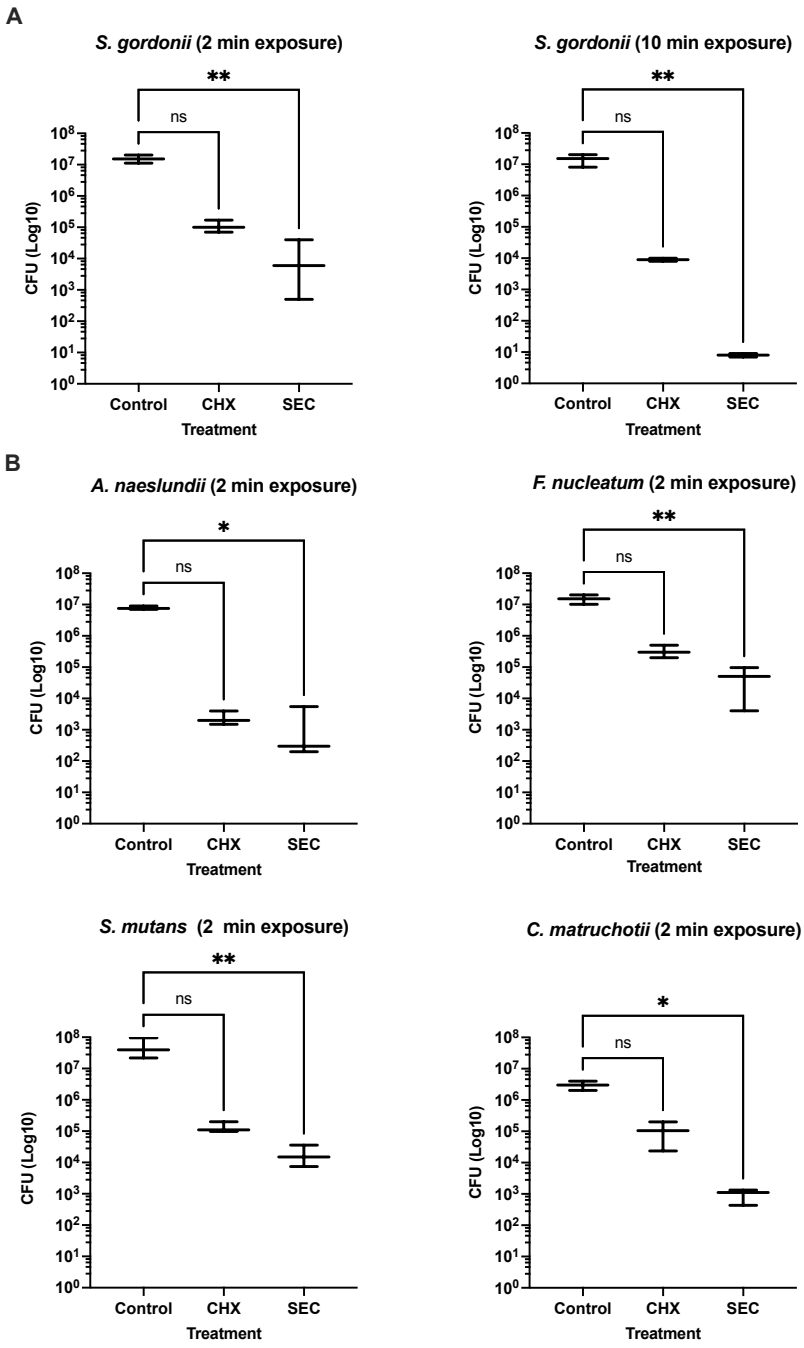
409 **Figure 1**



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411 **Figure 1.** (A) Viable counts of *S. gordonii* biofilms following exposure to chlorhexidine

412 (CHX) and (SEC) dentifrices for 2 or 10 minutes. (B) Viable counts of *F. nucleatum*, *C.*

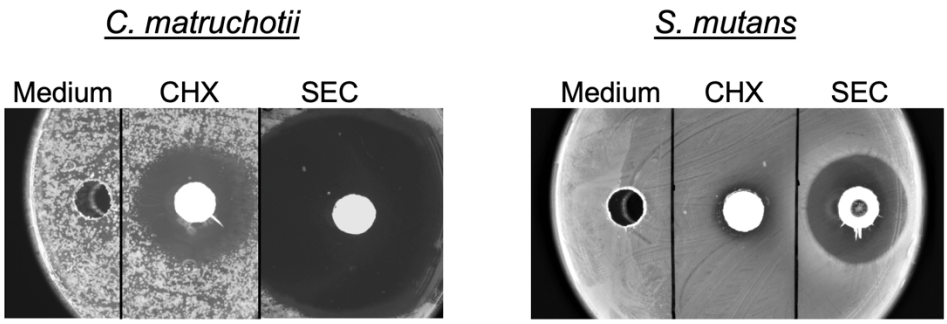
413 *matruchotii*, *S. mutans* and *A. naeslundii* biofilms following 2 min exposure to CHX and SEC

414 dentifrices. * = P <0.05 and ** = P <0.01 in Kruskal-Wallis test with Dunn's test for multiple

415 comparisons.

Figure 2

A



B

Treatment	*S. gordonii*	*S. mutans*	*A. naeslundii*	*F. nucleatum*	*C. matruchotii*
CHX	19 (+/- 1.4)	13 (+/- 0)	12 (+/- 0)	20 (+/- 2.8)	26.5 (+/-0.7)
SEC	34.5 (+/- 0.7)	25 (+/- 1.4)	35.5 (+/- 4.9)	56.5 (+/- 2.1)	48 (+/-4.2)

Figure 2. Well diffusion assay to assess susceptibility to CHX and SEC dentifrices. (A) Representative images showing halos of inhibition for *C. matruchotii* and *S. mutans*. (B) Halo sizes from 3 replicate experiments showing average diameter in mm +/- variance. The more intense red colour indicates larger halo size.

Figure 3

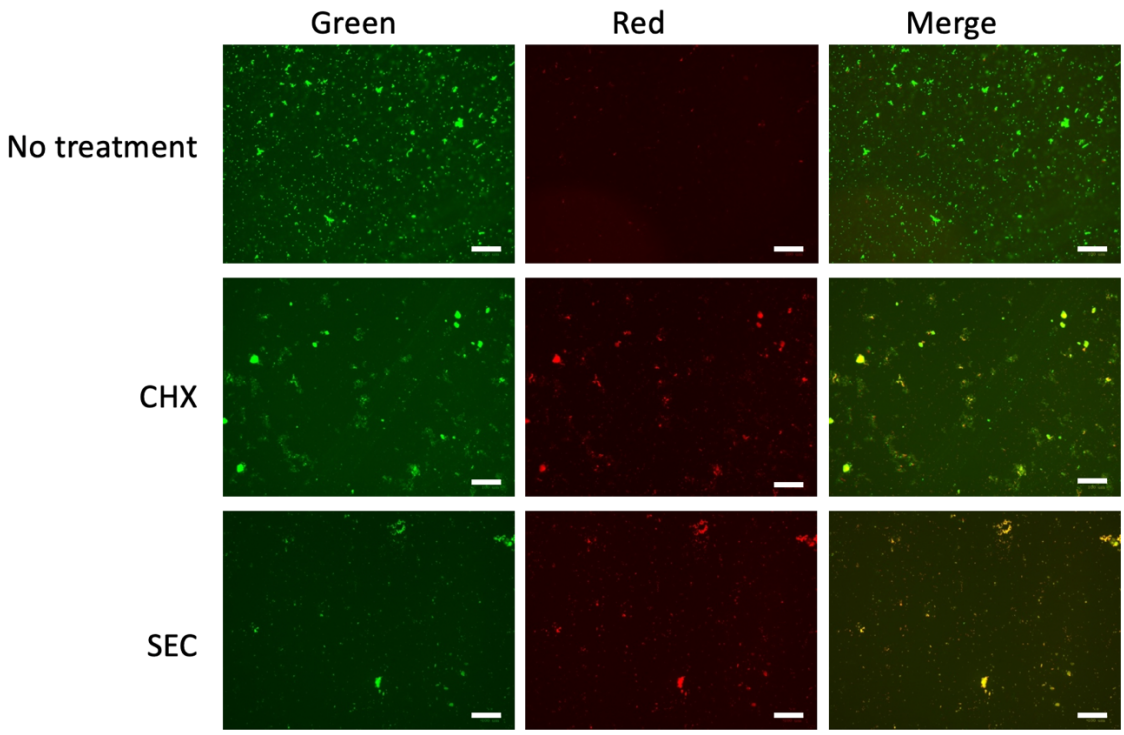


Figure 3. Qualitative assessment of biofilm viability and removal was carried out by staining biofilms with the LIVE/DEAD Baclight bacterial viability kit (Thermofisher). A tri-species biofilm of *S. gordonii*, *S. mutans* and *C. matruchotii* was grown and exposed to DMEM (control) or to a 30% v/v suspension of CHX or SEC dentifrice for 2 minutes. Biofilms were observed using a Zoe fluorescence microscope (BioRad). White bar corresponds to 100 µM.