Effect of Fluoride-Containing Toothpastes on Enamel Demineralization and *Streptococcus mutans* Biofilm Architecture

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Sodium fluoride (NaF) and stannous fluoride (SnF₂) are commonly incorporated into toothpaste formulations as a source of fluoride [Lippert, 2013]. An early clinical study evaluating NaF-containing toothpaste was unable to show an anticaries effect due to incompatibility issues with other components in the formulation [Bibby, 1945]. Some years later, SnF₂-containing toothpaste was shown to be clinically efficacious for caries reduction [Muhler et al., 1954, 1955], but it also had issues with stability (relating to the amount of soluble fluoride available over time) and tooth staining [Makin, 2013]. Since those early years, toothpaste formulations have changed considerably to become stable, using reliable vehicles for fluoride delivery, and also to deliver other compounds with potential health and cosmetic benefits [Zero, 2006; Stamm, 2007]. Thus, it is recognized that active compounds within a dentifrice formulation could have a different level of efficacy than when pure because other ingredients in the product could alter the bioavailability or activity of the active compounds [Zero, 2006; Lippert, 2013].

Evidence has demonstrated that modern fluoridated toothpastes, including among others either NaF- or SnF₂-based toothpastes, are effective at controlling dental car-
ies [Walsh et al., 2010] because of fluoride’s activity in reducing demineralization and enhancing remineralization [ten Cate, 2013; Tenuta and Cury, 2013]. Beside the caries control effect, SnF₂-containing toothpastes have been reported to reduce dental plaque [Sharaf et al., 2013], bleeding [Gerlach and Amini, 2012], halitosis [Farrell et al., 2008], erosion [Bellamy et al., 2014] and dentin hypersensitivity [Parkinson et al., 2013]. Although there is not enough clinical evidence to demonstrate that SnF₂-based toothpaste is more effective than NaF-based toothpaste for caries control [Marinho et al., 2003], it has been suggested that SnF₂ toothpaste could be superior [Faller et al., 1995]. This is because stannous has been indicated to have antimicrobial activity [Mayhew and Brown, 1981; Camosci and Tinanoff, 1984] and can reduce acid and glucan production [Ferretti et al., 1982; Zameck and Tinanoff, 1987]. On the other hand, NaF has been shown in vivo [Svanberg and Rolla, 1982] and in vitro [Fernández et al., 2016] to lack a substantial antimicrobial effect when simulating mouthrinse or toothpaste use. However, the contribution of the antimicrobial/anti-biofilm activity of stannous, as part of an SnF₂-containing toothpaste on the caries process, is not well understood. Thus, the aim of this study was to use an in vitro biofilm model system to evaluate the effect of a currently marketed SnF₂-containing toothpaste and a currently marketed NaF-containing toothpaste on biofilm architecture and enamel demineralization. To achieve this aim, we used a custom-built in vitro static biofilm system that paired with a confocal laser scanning microscope (CLSM) and used bovine enamel specimens that were amenable to enamel surface hardness testing.

Materials and Methods

An in vitro multi-day study was conducted using a modified Streptococcus mutans cariogenic biofilm model (fig. 1) that is based upon a previously validated model to study antimicrobials [Ccahuana-Vásquez and Cury, 2010] and fluoride [Fernández et al., 2016]. For this study five independent assays were made, each one in quadruplicate (total n = 20/group). Samples were coded and analyzed blindly. Bovine enamel specimens (4 × 4 × 1 mm) were prepared and analyzed for surface hardness (see Enamel Demineralization). Specimens of predetermined surface hardness (328 ± 13 HV; n = 120) were randomized in the experimental groups. The assembled model was sterilized using ethylene oxide before use.

Long-Duration (144 h) Biofilm Model System

The model employed in this study (fig. 1) used bovine enamel specimens that were assembled on acrylic holders bonded to the lid of 24-well culture plates. Each holder supported one specimen per well (fig. 1a). Specimens were incubated in sterile human saliva for 30 min to promote the formation of acquired pellicle and then suspended in brain heart broth (BHI) containing S. mutans UA159 inoculum. The inoculum was prepared by growing the strain for 18 h in BHI at 37 °C in an anaerobic gas jar. The suspension was subsequently diluted 1/500 in BHI supplemented with 1% sucrose. The specimens were incubated with the inoculum for 8 h at 37 °C in 5.5% CO₂. The medium was then exchanged for fresh BHI. After 24 h, the samples were exposed to 10% sucrose for 5 min 3 times a day and the BHI was replaced twice every day (online suppl. fig. 1; see www.karger.com/doi/10.1159/000444888 for all online suppl. material). The pH of the spent media was utilized as an indirect indicator of the biofilm acido-genicity. After 48 h of biofilm growth, the treatments were performed cyclically with 1 min exposure twice a day. The treatments were: (1) deionized water (negative control), (2) slurries at 33% w/v (1 part toothpaste: 2 parts water) or 15% (1 part toothpaste: 6 parts water) concentration of toothpaste containing either SnF₂ (Crest Pro-Health®, 0.454% SnF₂) or NaF (Crest Cavity Protection® 0.243% NaF) or (3) chlorhexidine (CHX) gluconate 0.12% (PerioGard®) (positive/antimicrobial control). After sucrose and treatment exposition, the samples were rinsed 3 times in saline solution (0.9% NaCl). At the end of each experiment, the specimens were collected to evaluate the biofilm structure and surface hardness of the enamel (fig. 1b).

Biofilm Staining, CLSM and Image Analysis

Following the removal of the specimens from incubation wells (fig. 1b), biofilms were stained with Live/Dead® reagent (Invitrogen, Carlsbad, Calif., USA) for 45 min at room temperature and washed twice in PBS (pH 7.4). Subsequently, five representative biofilm image stacks per specimen were taken using an inverted Leica SPE CLSM (Leica, Germany) equipped with a 40× 1.25 NA HCX PL APO infinity-corrected oil objective (fig. 1c). In order to derive biofilm architecture, IMARIS software (Bitplane, Zurich, Switzerland) was used to render images in 3D. Specifically, this was performed by using the Surpass visualization software component and applying a light source to the 3D reconstructed biofilm using shadow projection to generate defined edges and shadows. Biomass and average thickness were calculated using COMSTAT2 software [Heydorn et al., 2000]. Using the approach of Nance et al. [2013], cell viability was calculated by determining the percentage of green pixels (from the total of green and red pixels) in each image stack using ImageJ software [Collins, 2007].

Enamel Demineralization

Surface hardness was assessed using a Vickers microhardness test (Leco Corp., St. Joseph, Mich., USA). Four indentations (200 g for 10 s) at a distance of 200 μm from each other were created before and after treatments to calculate the percentage of surface hardness loss (%SHL) [Ccahuana-Vásquez and Cury, 2010].

Fig. 1. Photographs showing the design and use of the biofilm model system. a Enamel specimens assembled in an acrylic holder were attached to the lid of a 24-well culture plate. b After each experiment (144 h) the enamel specimens with the formed biofilm were separated from the lid, individually stained and positioned in a 96-well glass plate for imaging. c An inverted CLSM was used to take 5 images of the biofilm formed on each specimen.
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Statistical Analysis
All analyses were conducted using SPSS software (IBM Corp., version 20.0, Armonk, N.Y., USA) with 5% significance. Statistical analyses of %SHL were performed using ANOVA and Tukey’s tests. Imaging data were analyzed by Kruskal-Wallis and Mann-Whitney U tests.

Results
The %SHL, as an outcome following different treatments, is presented in figure 2. As inferred by the %SHL, all the treatments resulted in a significant reduction in demineralization. The least amount of demineralization was observed for biofilms that underwent treatment with 33% slurry of SnF2-containing toothpaste or the 0.12% CHX (6.9 and 4.9 %SHL, respectively). Treatments with the NaF-containing toothpaste also resulted in a significant decrease in %SHL compared to the negative control, but these were not as dramatic as the SnF2 treatments (fig. 2). Treatment with different concentrations of SnF2-containing toothpaste resulted in statistically significant differences in %SHL (slurries at 33% SnF2 vs. 15% SnF2), while treatment with NaF-containing toothpaste showed a numerical trend, but this was not statistically significant (p = 0.15). Taken collectively, the data indicated that SnF2-containing toothpaste was approximately twice as effective as NaF at reducing %SHL.

The antimicrobial/antibiofilm effects of the different treatments on the S. mutans biofilms was determined by analyzing images obtained by CLSM. A summary of the

![Graph](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAA3wAAAaCAYAAAAf150cAAAAGXRFWHRTb2Z0d2FyZQBBZG9iZSBJbWFnZVJlYWR5ccllPAAAAFmJREFUeNrs6ZQr4B1Ox59s8hOCAMJ9wA6kMLQAAAABJRU5ErkJggg==)

Fig. 2. %SHL. Slurries of SnF2 or NaF were prepared mixing toothpaste and deionized water at 33% w/v (1 part toothpaste:2 parts water) or 15% (1 part toothpaste:6 parts water). Values are means ± SD (n = 20). Different lower-case letters represent statistical differences among treatments (p < 0.05).

![Images](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAA3wAAAaCAYAAAAf150cAAAAGXRFWHRTb2Z0d2FyZQBBZG9iZSBJbWFnZVJlYWR5ccllPAAAAFmJREFUeNrs6ZQr4B1Ox59s8hOCAMJ9wA6kMLQAAAABJRU5ErkJggg==)

Fig. 3. Representative 3D reconstruction of images obtained using CLSM of the treated biofilms and results of architecture and viability. Slurries of SnF2 or NaF were prepared mixing toothpaste and deionized water at 33% w/v (1 part toothpaste:2 parts water) or 15% (1 part toothpaste:6 parts water). Values are means ± SD (n = 20). Different lower-case letters represent statistical differences among treatments (p < 0.05).
results are presented in figure 3. The data indicated that treatment with SnF$_2$-containing toothpaste resulted in a dose-dependent response for all three parameters analyzed. In particular, computational image analysis highlighted that SnF$_2$-containing toothpaste treatments (slurries at 33 or 15%), as well as CHX treatment, caused a significant decrease in biomass and thickness compared to the negative control. This was confirmed by 3D computational rendering of individual images in the confocal stacks and showed that the SnF$_2$-containing toothpaste and CHX treatments caused biofilm architecture to be more archipelago-like, as opposed to confluent biofilms that developed following the other treatment exposures (fig. 3). Viability analyses indicated there was a modest but statistically significant decrease in the viability of _S. mutans_ biofilms treated with SnF$_2$-containing toothpaste compared to the untreated biofilms. NaF-containing toothpaste did not show as obvious concentration-dependent changes in biomass and thickness, and only a small reduction in bacteria viability.

The pH in the culture medium (fig. 4), measured as an indirect indicator of biofilm acidogenicity, was similar for all groups before starting the treatments. After the first treatment (48 h of biofilm development; fig. 4), a clear treatment effect was initially observed, especially for CHX and slurries at 33 and 15% of SnF$_2$-containing toothpastes, but this was curtailed by 72 h of biofilm development. Following this period, the pH of the culture medium was sustained at around 5 for all groups except for those treated with the 33% slurry of the SnF$_2$-containing toothpaste, which was approximately 5.5, and the CHX group which was approximately 7.0 (p < 0.05).

![Fig. 4](image_url)

**Fig. 4.** The change in pH of the culture medium at different times during the incubation period. Slurries of SnF$_2$ or NaF were prepared mixing toothpaste and deionized water at 33% w/v (1 part toothpaste:2 parts water) or 15% (1 part toothpaste:6 parts water).

Values are means ± SD (n = 20). Different lower-case letters represent statistical differences among treatments at each time point (p < 0.05).
Discussion

Biofilm-based laboratory models are essential for studying anticaries therapies because biofilms are a primary factor for caries development [Marsh, 2006], and their presence can influence treatment outcomes [Zhang et al., 2015]. Several biofilm model systems have previously been used to evaluate the effect of oral health care products and active ingredients on oral biofilms [McBain, 2009; Ledder and McBain, 2012; Nance et al., 2013; Zhang et al., 2015]. These systems, however, do not typically combine approaches and technologies to obtain a detailed architectural analysis of biofilms grown on enamel, and typically have limited accessibility to explore the effects on dental surfaces [McBain, 2009; Valappil et al., 2014]. For this study, a custom-built, low-cost and small footprint in vitro biofilm model system (fig. 1) allowed us to explore the effect of different toothpastes on S. mutans biofilms and enamel demineralization. We were able to show that the model can discern and measure the effects of different commercial toothpaste formulations, since both SnF₂ and NaF toothpastes reduced enamel demineralization, but only the SnF₂-containing toothpaste caused appreciable changes in biofilm architecture.

The difference in the antimicrobial/antibiofilm effect between the SnF₂- and NaF-containing toothpastes cannot be attributed to differences in fluoride concentration, since both have similar levels (0.11% fluoride). Because the antibacterial activity of stannous ions is well known [Mayhew and Brown, 1981; Camosci and Tinanoff, 1984; Zameck and Tinanoff, 1987], its presence in the SnF₂-containing toothpaste suggests that it is the responsible ingredient for the antimicrobial/antibiofilm effect observed with this formulation (fig. 3). However, it cannot be discarded that other ingredients present in the commercial product could have contributed to these findings. The greater effect of the SnF₂ toothpaste on the reduction of enamel demineralization (%SHL; fig. 2) is therefore potentially due to the antimicrobial/antibiofilm effect, resulting in a reduced cariogenicity of the biofilm and an enhanced physicochemical effect of the fluoride against dental caries [ten Cate, 2013]. SnF₂-containing toothpaste treatments created archipelago-like biofilm coverage (fig. 3) that hypothetically could allow SnF₂ to have a more direct access to the exposed enamel surface. Furthermore, Zhang et al. [2015] recently showed in a long-term biofilm model that the presence of a biofilm is able to limit the anticaries efficacy of NaF, perhaps also partly explaining the lower effect of the NaF-containing toothpaste in this experiment. Nevertheless, this hypothesis needs to be further tested using appropriated controls for the commercial SnF₂- and NaF-containing toothpastes (formulated identical to the commercial ones, but without the fluoride salts). From an experimental design perspective, studying commercial toothpastes, instead of using pure SnF₂ and NaF salts, allowed us to study these active salts in the form used by the public, giving us insights for potential ‘real-world’ outcomes. This is relevant because toothpastes contain complex formulations that could alter the bioavailability of NaF or SnF₂ [Zero, 2006; Lipert, 2013].

Compared to the untreated biofilms, the architecture and biomass of both the biofilms treated with SnF₂-containing toothpaste and the CHX-treated biofilms were strikingly different to the controls (fig. 3). The biofilms treated with NaF-containing toothpaste were more similar to the controls. For all treatments, BacLight staining indicated that the biofilms were viable. Given that the last treatment prior to imaging was done 16 h earlier (online suppl. fig. 1), the general lack of dead cells in the biofilms is not necessarily surprising because dead cells were likely to have been replaced by remaining viable cells during this period. This would change the ratio of the green to red (Live/Dead) signal and thus mask the actual reduction in cell numbers and killing as a consequence of the treatment. Indeed, the architectural and biomass differences suggest that SnF₂-containing toothpaste and CHX treatments had long-term sustained effects on biofilm development. Such architectural and biomass changes were probably due to an antimicrobial/antibiofilm effect. Given the strong antimicrobial nature of CHX, we hypothesize that the decrease in biomass and altered biofilm architecture was due to repeated bacterial killings and subsequent effect on regrowth. Regrowth could have been slowed down by residual CHX bound to dead cells and enamel [Baca et al., 2012; Babu and Garcia-Godoy, 2014]. Stannous can alter S. mutans growth and metabolism [Mayhew and Brown, 1981; Camosci and Tinanoff, 1984] as well as inhibit bacterial adhesion/plaque formation and acid production [Tinanoff et al., 1976; Skjorland et al., 1978], potentially retarding biofilm development and altering its architecture, as observed in our system (fig. 3).

The effect of SnF₂-containing toothpaste on the biofilm architecture and biomass of the biofilms, as well as on the reduction of enamel demineralization, correlated with the results of the culture medium pH (fig. 4). The neutral pH measured in the culture medium of the CHX-treated group (the antibacterial/positive control) indicates that biofilm cells were not producing enough acid to cause a pH drop. This is in agreement with the mechanism of action of CHX, which effectively inactivates bac-
teria by targeting membrane and cellular components [Jones, 1997; Gilbert and Moore, 2005]. Biofilms treated with slurry at 33% of SnF$_2$-containing toothpaste showed less acidogenicity than the other groups, which corresponds with the changes produced in the biofilm. This can also be explained by fewer bacteria per biofilm area to metabolize sucrose, and consequently less pH reduction. On the other hand, biofilms treated with NaF-containing toothpaste did not have an effect on $S$. mutans biofilm acidogenicity, which is in agreement with previous studies showing that the fluoride ion has limited antibacterial effect at the concentrations delivered in these products [Svanberg and Rolla, 1982; Ccahuana-Vásquez and Cury, 2010; Fernández et al., 2016].

For an appropriate interpretation of the results from this study, certain model-specific and experimental design matters should be considered. While the model system is static, it can be envisioned that this represents some in vivo anatomical regions between teeth that have limited exposure to mechanical forces and salivary flow – sites where caries lesions are frequently developed [Nyhad et al., 2008]. While the system only used a single-species biofilm, the model is useful because the inclusion of $S$. mutans created a cariogenic condition to produce caries-like lesions in enamel, and this allowed us to study changes in demineralization and on biofilm architecture. The treatment of biofilms with 0.12% CHX was included in the study as a positive/antimicrobial control and not as a positive control for caries. The antibacterial effect observed by CHX resulted in a sustained neutral pH of the culture medium, and consequently no enamel demineralization was expected. This observed reduction of demineralization is probably because this is a short-term model system and bacterial renewal/recolonization did not occur at the same rate as that in the oral cavity. However, the model differentiated the antiplaque (antibacterial) effect of the commercial SnF$_2$ toothpaste with the NaF-containing toothpaste. Nevertheless, further research should be conducted using a long-term flowing biofilm model to confirm our findings.

In conclusion, the biofilm model system enabled the evaluation of the effect of two different commercial toothpastes on enamel and the supported biofilm architecture. The evaluated NaF and the SnF$_2$-containing toothpastes showed a concentration-dependent reduction of enamel demineralization. The SnF$_2$-containing toothpaste was particularly effective at reducing biofilm biomass and altering biofilm architecture in this model. The SnF$_2$-containing toothpaste also showed a greater ability to reduce enamel demineralization, which could be explained by the observed antimicrobial/antibiofilm activity.

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**Author Contributions**

C.E.F., C.G.-C. and A.H.R. conceived and designed the experiments. C.E.F. performed the experiments. C.E.F. and C.G.-C. analyzed the data. C.E.F. and C.G.-C. drafted the paper. The paper was reviewed by C.E.F., M.F., D.S., J.A.C., A.H.R. and C.G.-C.

**Disclosure Statement**

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