**TITLE:** A commercial SnF2 toothpaste formulation reduces simulated human plaque biofilm in a dynamic typodont model

**RUNNING TITLE:** Stannous fluoride and oral biofilm typodont model.

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Sara Palmer: Contributed to conception, design, and critically revised the manuscript.

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All authors gave their final approval and agreed to be accountable for all aspects of the work.

**Abstract (248 words)**

Aims: We present a dynamic typodont biofilm model (DTBM) incorporating 1) human dentition anatomy, 2) fluid flow over intermittently fluid bathed tooth surfaces and 3) an oxic headspace to allow aerobic and anaerobic niches to develop naturally, as a screening tool to assess the effect of stannous fluoride (SnF2) toothpaste against a simulated human plaque biofilm (SPB). Methods and results: First, hydroxyapatite (HA) coupons were inoculated with human saliva/plaque and cultured at 37oC under air. Selected species representative of common commensal and anaerobic pathogens were quantified for relative abundance changes over 4d by PCR densitometry to confirm the culture conditions allowed the proliferation of these species. A continuous culture DTBM reactor on a rocker table was inoculated with saliva/plaque and incubated at 37°C for 24h. Tooth shear stress was estimated by particle tracking. A SnF2 toothpaste solution, or a sham rise was administered twice daily for 3d to mimic routine oral hygiene. SPB biomass was assessed by total bacterial DNA and methylene blue (MB) staining. Early colonizer aerobes and late colonizer anaerobes species were detected in the HA and DTBM, and the trends in changing abundance were consistent with those seen clinically. Conclusions: Treatment with the SnF2 solution showed significant reductions of 53.05% and 54.4% in the SPB by MB staining and DNA, respectively. Significance and impact of study: The model has potential for assessing dentition anatomy and fluid flow on the efficacy of antimicrobial efficacy against localized SPB and may be amenable to the plaque index clinical evaluation.

**Introduction**

Background

Dental plaque biofilms are complex ecosystems formed from hundreds of interacting species where microorganisms are bathed in saliva and gingival crevicular fluid ([Halib et al. 2019](#_ENREF_5)). *In vitro* model biofilms are useful as a screening tool for assessing efficacy of antimicrobial dentifrices under controlled conditions. Conventionally *in vitro* simulated plaque biofilms are grown on flat surfaces such as hydroxyapatite discs or glass ([Kolderman et al. 2015](#_ENREF_11); [Halib et al. 2019](#_ENREF_5)). To more realistically recreate the anatomy of the teeth, and how this may influence removal by brushing Ledder et al. ([Ledder et al. 2019](#_ENREF_13)) used a modified drip flow system in which liquid media was dripped onto typodont teeth to grow the biofilm. Training typodonts have also been used to recapitulate the physical anatomy of the mouth with respect to how water jets, brushing and antimicrobial dentifrices may remove a single species *Streptococcus mutans* biofilm ([Rmaile et al. 2014](#_ENREF_20); [Rmaile et al. 2015](#_ENREF_21)). Laboratory tools for assessing novel antimicrobial agents in toothpastes and mouthwashes to improve oral health are important as early screening phases in formulations development. However, many of these laboratory methods do not capture the shape and positioning of the teeth or the liquid flow around tooth surfaces, which are wetted but not completely submerged in the oral cavity. proposed to o

Here, we develop a continuous culture dynamic flow typodont model to assess the antimicrobial efficacy of stannous fluoride (SnF2) in a toothpaste formulation on the reduction of simulated human plaque biofilm in our model. We chose a SnF2 formulation to validate our model since SnF2 is a broad-spectrum antimicrobial agent widely used in oral care products for the control of dental plaque and treatment of gingivitis ([Tinanoff 1995](#_ENREF_30" \o "Tinanoff, 1995 #131)). More specifically, the stannous (II) ion (Sn [II]) has been established to be the bioactive species that exerts the antiplaque effect by decreasing bacterial biomass/virulence and inhibiting bacterial metabolism ([Tinanoff 1990](#_ENREF_29" \o "Tinanoff, 1990 #132); [Tinanoff 1995](#_ENREF_30" \o "Tinanoff, 1995 #131); [Bellamy et al. 2012](#_ENREF_2" \o "Bellamy, 2012 #133); [Parkinson et al. 2020](#_ENREF_17" \o "Parkinson, 2020 #134)). A meta-analysis of clinical data on the use of SnF2 from 2010 to 2019 showed that SnF2 significantly improved enamel wear loss compared to control groups and the authors conclude SnF2 as a dentifrice showed favourable promise compared to sodium fluoride, herbal toothpaste or triclosan products ([Fiorillo et al. 2020](#_ENREF_4" \o "Fiorillo, 2020 #3874)).

Before moving to the complexity of the typodont system we first grew simulated plaque biofilm on HA coupons and used PCR to determine if five key species representing primary colonizers and late anaerobic colonizers could establish in the biofilm over time, before moving to the more complex typodont model..

Aims

The aim of our study was to develop a typodont model (an adult human size model of the teeth and mouth) to incorporate a number of features recapitulating physical aspects of the oral cavity which we thought important for simulated plaque development. First, was to provide growth surfaces that accurately represented the size and analtomy of adult dentition. Second was a system in which the growth surfaces were not completely submerged but were constantly bathed in growth media and provided fluid flow around the tooth surfaces. Third to use an air headspace, rather than an anoxic headspace. For preliminary assessment of the utility of the model we grew simulated human dental plaque biofilms on a typodont in a continuous feed reactor positioned on a rocker table and assessed biofilm formation on individual teeth by simple crystal violet staining and total bacterial DNA on typodonts treated with a commercially available stannous fluoride toothpaste formulation compared to sham treated controls.

**Material and Methods**

***Growth media***

A modified brain heart infusion (M-BHI) broth was used for cultivations. BHI broth (Sigma Aldrich, USA) was supplemented with 5mg/L hemin (Alpha Aesar, USA), 1mg/L menadione (MP Biomedicals, LLC, France), 0.1g/L L-cysteine (Sigma, USA) and 1g/L yeast extract (Sigma, USA).

***Conventional PCR and densitometry for semi-quantitative identification of target species in the saliva/plaque inoculum and simulated plaque biofilm (SPB)***

PCR conditions were optimized to identify five target species using the following strains *V. parvula* 17745*, S. oralis* 10557, *A. viscosus* 43146*, F. nucleatum* 10953 *and P. gingivalis* 33277. Due to uncertainty of taxonomic identification of *A. viscosus* with respect to identification of this species in human strains we denote this species in quotation marks ([Könönen and Wade 2015](#_ENREF_12)). DNA extraction, primer sets, and PCR conditions are described in Supplemental Information and Supplemental Table 1. Gel densitometry analysis was performed for semi-quantification using NIH FIJI image analysis software ([Schindelin et al. 2012](#_ENREF_23)), where the brightness of the band was measured by positioning a region of interest (ROI) of consistent area within each band and measuring the grayscale value. The greyscale of pixel intensity ranged from 0 (black) to 255 (white). The pixel intensity of the background was measured by positioning the ROI to the left of the ladder (i.e., a blank lane) at the corresponding position on the gel where the expected band size base pair (bp) would occur from extrapolation of bands in the positive lanes.

***Selective medium for the isolation and presumptive identification of P. gingivalis***

Because our biofilm was developed under an oxic headspace we were particularly concerned that obligate anaerobes might not establish in the biofilm therefore we used selective culture to confirm that anaerobes were present and viable in our SPBs. *P. gingivalis* agar AS-6422 (Anaerobe Systems, USA) was used as an enriched selective medium for the isolation and identification of *P. gingivalis*. The coupon was transferred to an anaerobic chamber and placed in a 50mL falcon tube (Falcon, Thermo Fisher Scientific, Waltham, MA, USA) with deaerated PBS. The tube was sealed tightly and brought outside the chamber for sonication and vortexing. The tube was returned to the anaerobic chamber for plating. The obligate anaerobes *Prevotella* and *Porphyromonas* spp. produce black pigmented colonies on this agar. PCR was run to confirm *P. gingivalis* on selective agar plates.

***Collection of human saliva / plaque inoculum***

Plaque was removed using a toothbrush from teeth and tongue with no toothpaste from healthy adult donors using an adapted protocol ([Nance et al. 2013](#_ENREF_14)). The participant had not taken antibiotics for at least 3 months prior to donation. Donors were asked not to eat anything or practice oral hygiene 8 h before collection. Briefly, plaque was recovered using a manual toothbrush (Kids, Oral BSAP-No: 80292664) from the teeth and tongue by brushing with no toothpaste. The toothbrush head was vortexed (Vortex-Genie® 2 mixer, Scientific Industries, Inc, Bohemia, N.Y., USA) in 10 mL Phosphate-Buffered Saline (PBS, Gibco, Thermo Fisher Scientific) for 3 min to transfer the plaque from the brush to the PBS. Vortexing was conducted in an anaerobe chamber (Bactron, USA, with a 5% CO2, 5% H2 and 90% N2 headspace. The bacteria were pelleted by centrifugation (10 G for 3 min) and resuspended in the pooled saliva. Glycerol was added to a final concentration of 25%. Aliquots were stored in 1.5 mL cryogenic tubes (Thermo Fisher Scientific, USA) at −80 °C. This study was approved by the OSU IRB (protocol 2017H0016) with written informed consent.

***Hydroxyapatite Coupon Model***

First, to ensure our media and oxic headspace could support the development of a microbial community representative of those found clinically we grew SPB on HA coupons in a relatively simple system before moving to the more complex typodont model. HA coupons (1.25 cm in diameter, surface area of 2.7 ± 0.2 cm2, (non-sintered, Hi-Med, Old Bethpage, NY) were placed into each well of a 12 well plate (Falcon, corning, USA). 2 mL of sterile of M-BHI was added to each well followed by 500 µL of the saliva/plaque inoculum and incubated at 37 oC (Thermo Fisher Scientific) under air. Biofilms were grown for 4 days with daily media exchange. Each day a triplicate set of coupons were sacrificed for DNA 16S RNA gene phylogenetic analysis and one coupon was sacrificed for MB staining.

***DNA extraction***

The HA coupons were transferred to 50 mL tubes (Falcon, Thermo Fisher Scientific, USA) with 5 mL of sterile phosphate-buffered saline PBS. Biofilm was removed by sonicating in a sonicator bath (Model # 97043-964, VWR International, West Chester, PA, USA) for 3 min. The sonicate was centrifuged (Legend micro 21, Thermo Fisher Scientific, USA) at 10 G for 10 min. The supernatant was discarded, and the pellet used for DNA extraction. DNA was extracted using a boiling method ([Khosravi et al. 2014](#_ENREF_9)), a simple and cheap method that has been shown to be effective for human dental plaque ([Nance et al. 2013](#_ENREF_14)). The biofilm was boiled in dH2O (Invitrogen, USA) for 10 min and then chilled for 2 min at 20 °C. The sample was then centrifuged at 16 G for 10 min at room temperature.

***Conventional PCR and densitometry for semi-quantitative identification of target species***

Amplification was performed in a 25 µL mixture containing Mg2+, dNTPs, and recombinant Taq DNA Polymerase at concentrations sufficient for routine, 10µM forward and reverse primers (Supplemental Table 1) and 2 µL bacterial extract in a Bio-Rad PCR system thermal cycler. PCR was carried out: an initial denaturation step for 4 min at 94 °C, with 45 cycles of 30 s at 95 °C, 1 min at 58 °C and 30 s at 72 °C, followed by 5 min at 72 °C. Agarose gel (Sigma, USA) was prepared at a concentration of 1.5% (w/v) in 60 ml Tris-Borate Buffer (TBE). 1 µL of 10 µg/mL ethidium bromide (Sigma, USA) was incorporated into the gel for a final concentration of 0.5 µg/mL and electrophoresed at 90 V for 60 min. A low range DNA Ladder (O’GeneRuler) was used to size PCR products. Bands were visualized using a gel documentation system (ChemiDoc XRS, Bio-Rad, USA) under ultraviolet (UV) illumination. We had attempted to use RT-PCR to quantify the ratio of target species more accurately in the biofilm but unfortunately found that the boiling method extraction somehow interfered with the RT-PCR reaction (data not shown).

***Visualization of simulated plaque biofilm on HA coupons***

A coupon was removed daily and dip-rinsed with PBS to remove the planktonic cells. The coupon was stained with 1 mL 0.05% methylene blue (MB) for 30 sec, then rinsed twice to remove excess stain prior to photographing.

**The Dynamic Typodont Biofilm Model (DTBM)**

***Disinfection of the model***

We used a training typodont (Practicon, Greenville, NC). Since the typodont could not be autoclaved we used a chemical disinfection method. 150 mL of hydrogen peroxide (3% stabilized) for 20 min incubation and 150 mL of Colgate-Total mouthwash containing 0.075% cetylpyridinium chloride for 1 hr was added to the typodont and finally rinsed with dH2O (Invitrogen, USA). The disinfected typodont was placed inside a sterile beaker (500 mL) with 125 mL of sterile M-BHI broth and incubated for 24 hrs at 37 ºC to check for contamination. No evidence of growth by turbidity after 3 days incubation indicated that the typodont was sterile.

***Estimate of fluid flow and shear stress around the teeth during rocking in the typodont model***

To visualize flow around the teeth we used neutrally buoyant (1.00g/cc) blue polyethylene microspheres (180-212 µm diameter, Cospheric, CA, USA). The microspheres were first wetted properly by soaking in a hand soup solution for 2 hours prior to the flow study. A USB digital microscope (2.0 Megapixels, up to 230X magnification, Plugable, USA) and LED halo light with brightness adjustment control were mounted on the rocker with an observation stand so that the teeth appeared stationary while the liquid moved relative to the teeth. Movies were recorded at 20 fps. The velocity (u) of 13 individual beads close (within 3 mm) to the surfaces of various teeth were estimated by measuring the distance travelled over a known number of still frames using NIH image. The distance of the beads from the tooth surface (d) of the front incisor was estimated by eye from the movies using beads and features on the tooth as frames of reference. The shear rate (γ) was calculated from γ = u/d. The shear stress at the tooth surface (τw) was calculated from τw = ɲγ, where ɲ = absolute viscosity which we assumed was 0.75 cP (0.75 mPa.s or 7.5x10-4 Kg.m/s2) based on that of a 0.9% saline solution at 37oC ([Ozbek et al. 1977](#_ENREF_15)) to represent our media. Reynolds (Re) number was estimated from Re=ρuL/ ɲ where ρ = density which we assumed was 1000 kg/m3 and L was a characteristic length which we assumed was 1 cm (0.01 m, i.e. the approximate dimension of a tooth width). The Re was in the range of 10-100 suggesting a laminar flow regime. Therefore, our first approximation of τw assumed a linear velocity gradient from the no-slip zero flow condition at the surface of the tooth based on the small distances (mm’s) from the tooth surface.

***Inoculation and growth conditions***

The reactor system consisted of a 500 mL RDL310 Rotating Disc Ported Lid (BioSurface Technologies, Bozeman, MT) fitted with a bacterial vent filter (BST 02915 BioSurface Technologies, Bozeman, MT) (Fig. 1). One port was used to continuously deliver nutrient media, and another was used to draw off effluent using peroxide-cured silicone tubing with an inner diameter of 3.1mm (Cole-Parmer, Masterflex L/S16) with a peristaltic pump (Cole-Parmer, Ismatec IPC ISM932A, Barrington, IL) set at a flow rate of 0.25 mL/min. The disinfected typodont was placed aseptically into the autoclaved reactor. The effluent tubing was positioned to maintain a depth of liquid that was approximately level with the gum line, which gave a volume of 125 mL. The reactor was kept at 37°C using silicone heating tape (HSTAT051002, BriskHeat, Columbus, OH). 125 mL of fresh M-BHI broth was added. Next, 1000 µL pooled saliva/plaque was inoculated. The reactor system was placed on a rocker table (Bellco, USA) which supplied a front to back rocking motion with an angle of 62º. The teeth in the mandibular jaw were numbered according to the “universal numbering system” (Supplemental Fig. 1). When the front of the rocker was down the central incisors were submerged and the back 3rd molars were out of the media with the reverse when the front of the rector was upwards. The teeth were continually bathed in media but not continuously submerged. To minimize disruption to the biofilm during removal the screw was removed, and the pressure fit in the silicone gum socket was firm enough to keep them secured during rocking. Fresh media was pumped in at a flow rate of 0.25 mL/min. Triplicate typodonts were used in each run. To ensure the typodont teeth could support SPB with representative aerobic and anaerobic species we ran the system for 4 days with no treatment. On day 4 teeth 17 and 23 were extracted for DNA extraction and PCR as previously described. Each tooth was transferred in to 50 mL Falcon tubes with 5 mL of sterile PBS and dip-rinsed to remove the planktonic cells. Biofilm was removed by cell scraper and vortexing for 3 minutes. The supernatant was then centrifuged (Legend micro 21, Thermo scientific, USA) at 10 g for 10 minutes. The supernatant was discarded, and the pellet used for DNA extraction using the “boiling method” as previously described.

***Biofilm dentifrice treatment with the SnF***2 ***formulation***

To assess whether the model would be sensitive enough to identify differences in biofilm due to the exposure of antimicrobial dentifrices we exposed typodonts to a commercial toothpaste formulation (Colgate TotalSnF) containing 0.454% (0.15% w/v fluoride ion) in the form of a 1:5 (w/v) solution by dissolving 1 gram of toothpaste in to 4 mL of dH20 (Invitrogen, USA). We used a solution rather than whole toothpaste to allow equal exposure to the typodont surfaces without the need for physical brushing which may introduce variability and extended handling. We also wanted to isolate the action of the active ingredients on SPB in the typodont model from the physical action of brushing. When the toothpaste is used there is a dilution effect from tap water and saliva, thus the active ingredient would be diluted during routine brushing ([Satou et al. 2020](#_ENREF_22)). Application as a slurry has been shown clinically to improve efficacy, presumably due to increased access to hard-to-reach high risk sites for caries ([Sjögren 1995](#_ENREF_25)). However, a dilution greater than 1:3 was shown to reduce efficacy ([Satou et al. 2020](#_ENREF_22)). The final concentration of SnF2 in our toothpaste solution was 0.0908% (908 µg/mL). After 24 hrs of initial growth the typodont was removed from the reactor and dip rinsed in 150 mL PBS to remove loosely adhered cells. Then, the typodont was incubated in 150 mL of toothpaste solution for 2 min before rinsing with PBS and placing back in the reactor system with 125 mL of fresh M-BHI broth. This was repeated twice a day at an interval of 7-8 hrs to simulate an evening and morning oral hygiene regime. This was repeated on days 2, 3 and 4. After the last treatment on day 4 the typodont was incubated for a further 2 hrs in fresh M-BHI broth prior to sampling for biofilm quantification. A sham untreated control was exposed to the same regime except PBS was used instead of the toothpaste solution. To assess whether there was background staining of MB from the typodont material we performed a blank measurement by incubating a typodont with sterile media for 1 hour and then staining with 0.05% MB for 30 sec. followed by elution and absorbance quantification.

***Staining and imaging of the simulated plaque biofilm on the typodont***

On day 4, teeth from the right mandibular quadrant (number 25-32) were taken for staining with 0.05% MB as previously described ([Parry et al. 2017](#_ENREF_18)). Briefly, the typodont was first rinsed with PBS to remove loose cells. Then, incubated with 150 mL MB for 30 sec, and rinsed twice to remove excess stain. The teeth were then photographed in the typodont. Then each tooth was removed and transferred to individual 50 mL falcon tubes (Falcon, Thermo Fisher Scientific, Waltham, MA, USA) and 1 mL glacial acetic (Sigma, USA) was added to elute the MB for 30 min. Absorbance was measured at 570 nm with a spectrophotometer (GENESYS™ 20 Visible Spectrophotometer, Thermo Fisher Scientific). Absorbance was calculated per tooth and per cm2 using estimates of supragingival tooth surface area ([Kimura et al. 1977](#_ENREF_10)).

***Quantification of biofilm biomass by bacterial DNA***

Teeth from the left mandibular quadrant (numbers 17-24) were removed from the typodont and transferred to individual 50 mL falcon tubes with 5 mL of sterile PBS. Biofilm was removed by vortexing for 3 min and sonicating (Sonics, vibra cell) for 3 min. The supernatant was centrifuged at 10 G for 10 min. The supernatant was discarded and the pellet used for DNA extraction using the boiling method ([Khosravi et al. 2014](#_ENREF_9)). DNA concentration was calculated ng per tooth (DNA concentration x total volume of DNA) and also ng DNA per cm2 using estimates of supragingival tooth surface area ([Kimura et al. 1977](#_ENREF_10)).

***Statistical analysis***

Each experiment was run with independent triplicate typodont reactor systems with the inoculum taken from the same pooled stock. Since there were large differences in size and shape of each tooth, the tooth number as well as treatment (treated vs sham control) were selected as a variable factors for analysis. Statistical analysis was carried out based on two independent variables using two-tailed Student’s t-test and Anova two-Factor without Replication with P < 0.05 considered significant. Graphs were made using Prism 8.0 software (Prism, GraphPad Software, San Diego California USA).

**Results**

***Species development on biofilms grown on the HA coupons and typodont teeth***

The pooled saliva/plaque inoculum contained each of the target species representing early facultative commensal colonizers and later anaerobic pathogens (Supplemental Figs. 2 and 3). Simulated plaque biofilms grown on HA coupons appeared patchy on day 1 but after 4 days of growth were more uniform and visibly thicker (Fig. 2a and 2b). Simulated plaque biofilm on the typodont teeth was also patchy and for the molars appeared denser on the occlusal surfaces (Fig. 2c). *S. oralis* showed a slight decrease in relative abundance after day 2 as the biofilm matured (Fig. 3 and supplemental Fig.4). *P. gingivalis* and “*A. viscosus”* were established in the biofilm after one day and showed an increasing trend in relative abundance as the biofilm matured.. *V. parvula* and *Fusobacterium* spp. signals were saturated (>255 grey scale pixel intensity) for all or some of the replicates or days, thus relative changes over time for these bacteria were not possible (Supplemental Fig. 4). These data demonstrated that all the target species were maintained in the biofilm and the anaerobic pathogens generally showed an increase as the biofilm matured. Furthermore, black colonies and PCR for confirmation (data not shown) confirmed the presence of *P. gingivalis* in the biofilm on days 2, 3 and 4 (Supplemental Fig. 3). All targeted bacteria species were identified in the untreated SPB after 4 days of growth (Supplemental Fig. 5).

***Biofilm treatment with the SnF***2 ***formulation***

The typodonts stained with MB prior to removing individual teeth in the SnF2 treated and control are shown in Fig. 4. MB eluted from the individual teeth the molars and premolars had greater biofilm than the incisors and canines (Fig. 5a). This was expected due to the larger surface area of these teeth and when normalized to tooth area the distribution more even (Fig. 5b). MB staining showed an average 53.0% reduction (P<0.0002) in biofilm biomass when treated with the SnF2 solution typodont compared to the control (P<0.0003) (Fig 5a&b). Total bacterial DNA showed a similar trend with greater biofilm on the molars and premolars (Fig. 5c) with an average percentage reduction of biofilm of 54.4% (P ≤0.001) (Fig. 5c) calculated per cm2. There was also a significant reduction of 53% for MB staining and a 54% reduction based on amount of DNA normalized for tooth area (P< 0.0001) (Fig. 5d).

***Estimate of fluid flow and shear stress around the teeth during rocking in the typodont model***

The particle tracking movies showed the beads moving in complex patterns around the teeth, through the interproximal spaces and through the grooves made by the cusps of the molars (Supplemental Movies 1-4). The average flow velocity of the 13 selected particle tracks was 3.0 mm/s ranging approximately between 1 and 10 mm/s and the shear stress ranged between approximately 4 and 40 Pa with an average of 14.3 Pa. The Re was in the range of 10 to 100 predicting laminar flow, however, the flow patterns of eddies around the teeth suggested flow separation caused by the interaction of the flow and the teeth as obstacles to flow. The fluid depth of the salivary film has been estimated to be 100 µm on average ([Collins and Dawes 1987](#_ENREF_3)) and fluid velocities have been estimated to range between 0.0013 and 0.0133 cm/sec and an average shear stress at the tooth surface of “~0.8 dyn/cm2” (0.08 Pa).

**Discussion**

We demonstrate the utility of a dynamic typodont biofilm model incorporating a number of features recapitulating the dentition of the oral cavity. We found that the rocking motion set up liquid flow around the tooth surfaces as would be expected *in vivo*. We estimated values of around 14 Pa, approximately 20 times higher than reported estimates ([Prakobphol et al. 1995](#_ENREF_19)) however these were made by averaging fluid production and tooth surface area of the whole oral cavity and liquid shear produced during tooth brushing are expected to be higher. While measuring the shear stress at specific locations in vivo is extremely difficult it is important to characterize flow in model systems since the flow rate will not only influence the forces acting on the biofilm influencing architecture and microbial community ([Hwang et al. 2014](#_ENREF_6)) ([Sharma et al. 2005](#_ENREF_24)) but also the exchange of nutrients, dentifrices and metabolites between the biofilm and the overlying fluid ([Stoodley et al. 2008](#_ENREF_26)). More complex particle imaging velocimetry (PIV) or computational fluid dynamics (CFD) may be employed to better characterize the flow conditions in the model. By varying the rocking speed it is possible to vary the fluid flow around the teeth to mimic flow during mouth-washing or tooth brushing. In the laminar range with a constant rocking angle, it is expected that the local velocity gradient will be directly proportional to the rocking speed. Under turbulent flow particle imaging velocimetry or computational fluid dynamics will be required to estimate local shear stresses.

Our aerobic growth condition allowed both facultative and obligate anaerobes to establish in the simulated plaque biofilm, consistent with James et al ([James 2012](#_ENREF_7)) who used a drip flow biofilm reactor to grow subgingival and supragingival plaque-like biofilms on surfaces continually bathed with flowing media under air. On the HA coupons *P. gingivalis* was established in the biofilm after day 2 and showed an increase as the biofilm matured. Furthermore, we were able to identify *P. gingivalis* by selective culture demonstrating the presence of viable bacteria. Fusobacteria spp. showed a slight increase as the biofilm matured but had established as early as 1 day. All target species were maintained in the biofilm and the anaerobic species showed an increasing trend as the biofilm matured while *S. oralis* declined (Fig 3b). These trends are consistent with the development of human plaque biofilms which show greater abundance of oral streptococci initially followed by increasing abundance of anaerobic pathogens ([Teles et al. 2012](#_ENREF_28)) and is also consistent with our hypothesis that consumption of oxygen by facultative species creates anoxic conditions favoring proliferation of anaerobes as the biofilm matures. Although our PCR on a select group of targeted species suggests a relevant ecological succession in our static HA model further 16S based metagenomics community analysis is required to characterize the development of microbial ecology in the typodont model. We used a relatively simple method of boiling for DNA extraction followed by PCR and gel densitometry to semi-quantify the relative abundance of each target species over time in the HA model and to confirm their presence in the untreated day 4 typodont biofilm. However, this method does not allow abundance comparison between species. Further, for *V. parvula* and *F.* spp. we had saturated signals and could not make conclusions regarding relative changes after signal saturation. To overcome this limitation the samples could have been diluted. An alternative approach is to use qRT-PCR. We had attempted qRT-PCR but got no amplification product suggesting that boiling might have resulted in the production of a substance which poisoned the PCR reaction. A more sophisticated approach with respect to characterizing the microbial community in the typodont model would be to use 16S rDNA-based metagenomic analysis, however, this adds cost and complexity in terms of bioinformatic analysis.

We used two relatively simple quick and economical methods as metrics for assessing biomass. MB staining showed an average of 53.05% reduction in biofilm biomass per tooth for the Colgate TotalSF treated typodont compared to the sham control (untreated) (P<0.05) (Fig 4a & b) and total bacterial DNA showed a similar average reduction of 54.4% total biomass (P<0.001) (Fig 4c & d). Thus, there was good corroboration between these methods. Since our SPB assays were a snapshot in time 2 days after the initiation of SnF2 treatment it was not clear whether such reduction resulted from the treatment causing detachment, inhibition of subsequent growth or both. Live cell imaging using flow cells would be a good approach to address this question of the mechanism of action of SnF2 on SPB. We point out that the reduction that we saw with a 1:5 diluted liquid application might have been increased with full strength active agents in the form of a slurry ([Sjögren 1995](#_ENREF_25)). Repeated exposure to SnF2 has been shown to significantly reduce the total amount of biofilm development in multiple in vitro reactor systems (Ledder and McBain 2012; Ledder et al. 2010).

Although, we used a commercial SnF2 formulation to validate that the typodont SPB model could produce an expected reduction in biofilm we did not assess whether this model had greater predictive value that the simpler HA coupon model. The purpose of the work presented here was to assess whether the typodont model had potential for further development. We did find significant differences (P<0.05) in the amount of biofilm biomass on different teeth which can be attributed to factors such as differences in tooth surface area and features as well as exposure to fluid flow. Our methylene blue staining (Fig. 4) showed that biofilm was heterogeneously distributed on the typodont teeth. Interestingly, the fissures in the SnF2 treated typodont visually had lower levels of staining than the untreated typodont, possibly reflecting that the biofilm in this area was less tolerant to SnF2 or that SnF2 had somehow accumulated in these features, or as the particle imaging suggested, there was flow along the fissures between the cusps. Pits and fissures are prone to carries and are therefore features of interest that can be explored in the typodont model which is not possible with flat surfaces. In future work image analysis will be used to quantify biomass on individual teeth prior to methylene blue extraction in order to determine how reproducible the heterogeneity is and how well it correlates with the distribution of human plaque as assessed by disclosing solution and thus the influence of an antimicrobial agent on a simulated plaque biofilm could be assessed in a similar manner to as they would clinically ([Joiner 2007](#_ENREF_8)).

**Conclusions**

Simulated plaque biofilm (SPB) readily grew on the typodont tooth surfaces. The SPB was highly heterogeneous and tooth number, as well as treatment type, was an important variable factor due to differences in surface area and local physical and chemical microenvironments created by the flow patterns around the teeth. Six species representing both early and late colonizers established in the SPB, even though the biofilm was cultured under air, supporting our hypothesis that the SPB creates its own anoxic niches as they do *in vivo*.The SnF2 toothpaste formulation significantly reduced simulated plaque biofilms (SPB) compared to those exposed to sham control treatments. We conclude that our dynamic typodont biofilm model has potential as a screening tool to assess the efficacy of antimicrobial dentifrices and introduces the complexity of various physical parameters on a scale relevant to the adult oral cavity, as well as allowing the assessment of removal from localized individual tooth surface anatomy using contrast agents such as methylene blue.

**Consent for publication**

Not applicable.

**Competing interests / Conflicts of Interest**

PS is funded by Colgate-Palmolive. CAD and KS helped design the study. Colgate-Palmolive provided Colgate Total SnF in a blinded unmarked toothpaste tube.

**Data sharing**

Data sets will be made available on request.

**Figure Quality**

All figures are 300 DPI

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**List of Tables**

**Table 1:** Oligonucleotide PCR primer sets for various regions of the 16S rRNA gene used to identify target species and genera in the biofilm by densiometric gel electrophoresis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sequence of primer**  | **Target and abbreviation** | **Product size** | **Reference** |
| F, GTTGACAGCCGATGAAGAAGATGAAR, TTCTCAGCAAAAGTACCGTCCTCG  | *S. oralis* (So) | 81bp | ([Park et al. 2013](#_ENREF_16)) |
| F, ATGTGGGTCTGACCTGCTGC    R,CAAAGTCGATCACGCTCCG | *A. viscosus* (Av)\* | 96bp | ([Suzuki et al. 2005](#_ENREF_27)) |
| F, GGATAGATGAAAGGTGGCCTCTR, CCAACTAGCTAATCAGACGCAAT | *V. parvula* (Vp) | 72bp | ([Suzuki et al. 2005](#_ENREF_27); [Àlvarez et al. 2013](#_ENREF_1)) |
| F, CGCAGAAGGTGAAAGTCCTGTAT R, TGGTCCTCACTGATTCACACAGA  | *Fusobacterium.*spp.(Fspp) | 101bp | ([Suzuki et al. 2005](#_ENREF_27); [Àlvarez et al. 2013](#_ENREF_1)) |
| F, TAC CCATCGTCG CCTTGGTR, CGGACTAAAACCGCATACACTTG | *P. gingivalis* (Pg) | 126bp | ([Suzuki et al. 2005](#_ENREF_27)) |
| \*due to uncertainty of taxonomic identification of *A. viscosus* with respect to identification of this species in human strains we denote this species in quotation marks following Könönen ([Könönen and Wade 2015](#_ENREF_12)). |

**List of Figures**

**Figure 1: Dynamic Typodont Biofilm Model (DTBM)** A) Schematic showing the main components of the system. B) Side view of the water level when the rocker was tilted all the way forward then all the way back illustrating how the teeth were cyclically bathed in nutrient media.

**Figure 2:** **Progression of biofilm development on HA coupons.** Over the 4 days of growth the biofilm progressed in surface coverage on the coupon, becoming progressively more uniform by day 4. A)Non-stained photographs of the simulated plaque biofilms on HA coupons after 1, 2, 3 and 4 days of growth. b) Four-day HA biofilm stained with MB (blue). c) Biofilms grown on a typodont tooth stained with MB (blue) is shown for comparison demonstrating greater heterogeneity.

**Figure 3: Representative image of densitometry data for the SPB over time.** *S. oralis* showed a slight decrease from day 2, whereas “*A. viscosus”* and *P. gingivalis* showed a steady increase. *V. parvula* and *Fusobacterium* spp. signals were saturated (>255 grey scale pixel intensity) for all or some of the replicates or days, thus relative changes over time for these bacteria were not possible (Supplemental Fig. 4). The background grey level is indicated by the solid line. Mean and 1S.D. (n=3).

**Figure 4: Typodont biofilm staining by MB**. Simulated plaque biofilms grown on the typodont and then treated every 8 hrs for 4 days with A) sham control rinse, B) Colgate TotalSnF. On the last day, the typodont was removed and stained with MB (Blue). The less intense staining of the SnF2 treated typodont indicates less biofilm than the sham and was later quantified by elution from each individual tooth measurement by absorbance.

**Figure 5: Treatment with SnF**2 **formulation significantly reduces simulated biofilm plaque assessed by MB staining and amount of bacterial DNA per tooth.** a) MB staining showed that there was significantly less biofilm on the teeth treated with SnF2 compared to the sham control P<0.0002. The average reduction was 53.0%. There was greater staining on the molars and premolars which was expected due to the larger surface area of these teeth. b) MB data per tooth normalized for tooth area shows a more even distribution. The blank shows the background level of MB staining. c) DNA quantification showed a 54.4% reduction in biofilm by the SnF2 treatment compared to the sham control (P<0.001). Similar to the MB staining there was greater amounts of DNA on the molars and premolars. d) DNA amount per tooth normalized for tooth area shows a more even distribution.

**List of Supplemental Figures**

**Supplemental Fig. 1:** Tooth Number Chart. Dental practitioner view with the tooth names and numbers.

**Supplemental Fig. 2**: Presence of representative target species and genera in saliva/plaque inoculum. Gel electrophoresis of 16S amplicons from the in vitro biofilms showing the presence of the target species and genera. Lane 2-3: replicate of sample, N: Negative control (without DNA), lane 1: Positive control with DNA extracted from pure cultures of *S. oralis* 10557, *V. parvula* ATTC 17745, *F. nucleatum* ATTC 10953 and *P. gingivalis* ATTC 33277.

**Supplemental Fig. 3:** *P. gingivalis* growth on selective media in the saliva/plaque inoculums and on each of 4 days of biofilm growth. Black colonies (indicated by red circles) and confirmed by PCR (data not shown) demonstrate the presence of *P. gingivalis* in the HA biofilm on days 2, 3 and 4.

**Supplemental Fig. 4:** DNA densitometer showing the relative changes in each of the five target species in the SPB over 4 days of growth in the static HA model. Triplicate coupons (R1,2 and 3) were used for each of the days. The ladder is shown to the right of the gels and the expected PCR band is indicated in white text on the gel. Below each gel is a “plot profile” grey scale of pixel intensity ranging from 0 (black) to 255 (white). All species had signals above background indicated their prescence in the biofilm. Signals cut off at 255 are saturated.

**Supplemental Fig. 5:** Gel electrophoresis of 16S amplicons from the day 4 untreated typodontbiofilms showing the presence of the target species. Lane 1 is the positive control with DNA extracted from pure cultures of *S. oralis* 10557, *V. parvula* ATTC 17745, *F. nucleatum* ATTC 10953 and *P. gingivalis* ATTC 33277. The size of the expected DNA band (bp) is indicated in yellow. Lanes 2 and 3 and from DNA extracted from teeth 17 and 23 respectively. The DNA ladder in base pairs (bp) is shown on the right. All of the target species were present in the typodont SPB at day 4.

**List of Supplemental Movies**

**Supplemental Movie 1:** Top down movie view of flow visulaized by beads flowing around the dentition of the typodont during rocking cycles. The camera was mounted to the rocker so that it appears as if the typodont is stationary and the liquid is moving back and forth from the front to the back. The beads show that flow is highly complex and varies at different positions on the typodont. Although the velocity of the flow as measured by the bead displacment between frames is predicted to be laminar flow between and around the teeth forms complex swirls.

**Supplemental Movie 2:** Top down close up movie of fluid flow visualized by beads around teeth 26-22 at the front of the typodont where flow was perpindicular to the labial and lingual tooth surfaces.

**Supplemental Movie 3:** Top down close up movie of fluid flow visualized by beads around teeth 28-31, the molars and bicuspids on the right lateral side. Even though the direction of flow was more parralel to the lingual and buccal tooth surfaces, beads could be seen flowing through the interproximal spaces and along the fissure patterns formed between the cusps of the molars.

**Supplemental Movie 4:** Top down close up movie of fluid flow visualized by beads around the 3rd and 2nd molar on the left lateral side of the typodont showing flow around, between and over the crown of the teeth between the cusps.