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ORIGINAL ARTICLE

A commercial SnF_2 toothpaste formulation reduces simulated human plaque biofilm in a dynamic typodont model

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Abstract

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 (SnF_2) toothpaste against a simulated human plaque biofilm (SPB).

Methods and results: First, hydroxyapatite (HA) coupons were inoculated with human saliva/plaque and cultured at 37°C under air. Selected species representative of common commensal and anaerobic pathogens were quantified for relative abundance changes over 4 days 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 24 h. Tooth shear stress was estimated by particle tracking. A SnF_2 toothpaste solution, or a sham rise was administered twice daily for 3 days 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 SnF_2 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.

KEYWORDS

biofilm, dental, dentition model, stannous fluoride

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). In vitro model biofilms are useful as a screening tool for assessing the efficacy of antimicrobial dentifrices under controlled conditions. Conventionally in vitro simulated plaque biofilms are grown on flat surfaces such as hydroxyapatite discs or glass (Halib et al., 2019; Kolderman et al., 2015). To more realistically recreate the anatomy of the teeth, and how this may influence removal by brushing (Ledder et al., 2019) 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 of *Strep. mutans* biofilm (Rmaile et al., 2014, 2015). 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. To further simulate the environment experienced by the tooth surfaces, rather than completely immersing the typodont in nutrient media we used a rocker so that teeth were bathed on each rocking cycle and the motion generated liquid flow around the model dentition. We proposed to grow the biofilms under air rather than in an anoxic environment to allow the biofilm to develop its own oxic and anoxic niches as seen in ex vivo dental plaque biofilms (von Ohle et al., 2010).

Here, we develop a continuous culture dynamic flow typodont model to assess the antimicrobial efficacy of stannous fluoride (SnF_2) in a toothpaste formulation on the reduction of simulated human plaque biofilm in our model. We chose an SnF_2 formulation to validate our model since SnF_2 is a broad-spectrum antimicrobial agent widely used in oral care products for the control of dental plaque and treatment of gingivitis (Tinanoff, 1995). 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 (Bellamy et al., 2012; Parkinson et al., 2020; Tinanoff, 1990, 1995). A meta-analysis of clinical data on the use of SnF_2 from 2010 to 2019 showed that SnF_2 significantly improved enamel wear loss compared to control groups and the authors conclude that SnF_2 as a dentifrice showed favourable promise compared to sodium fluoride, herbal toothpaste or triclosan products (Fiorillo et al., 2020).

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 anatomy 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 met methylene blue and total bacterial DNA on typodonts treated with a commercially available stannous fluoride toothpaste formulation compared to sham-treated controls.

MATERIALS AND METHODS

Growth media

A modified brain heart infusion (M-BHI) broth was used for cultivations. BHI broth (Sigma Aldrich, USA) was supplemented with 5 mg/L hemin (Alpha Aesar, USA), 1 mg/L menadione (MP Biomedicals, LLC, France), 0.1 g/L L-cysteine (Sigma, USA) and 1 g/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* 17,745, *Strep.*

oralis 10,557, *Act. viscosus* 43,146, *Fus. nucleatum* 10,953 and *P. gingivalis* 33,277 (Table 1). Due to uncertainty of taxonomic identification of *Act. viscosus* with respect to the identification of this species in human strains we denote this species in quotation marks (Könönen & Wade, 2015). DNA extraction, primer sets and PCR conditions are described in Supplemental Information and Table S1. Gel densitometry analysis was performed for semi-quantification using NIH FIJI image analysis software (Schindelin et al., 2012), 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 50 ml falcon tube (Falcon, Thermo Fisher Scientific) 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). 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) 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% CO_2, 5% H_2 and 90% N_2 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

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, GTTGACAGCCGATGAAGAAGATGAA R, TTCTCAGCAAAAGTACCGTCCTCG	*Strep. oralis* (So)	81 bp	(Park et al., 2013)
F, ATGTGGGTCTGACCTGCTGC R, CAAAGTCGATCACGCTCCG	*Act. viscosus* (Av)[a]	96 bp	(Suzuki et al., 2005)
F, GGATAGATGAAAGGTGGCCTCT R, CCAACTAGCTAATCAGACGCAAT	*V. parvula* (Vp)	72 bp	(Àlvarez et al., 2013; Suzuki et al., 2005)
F, CGCAGAAGGTGAAAGTCCTGTAT R, TGGTCCTCACTGATTCACACAGA	*Fusobacterium* spp. (Fspp)	101 bp	(Àlvarez et al., 2013; Suzuki et al., 2005)
F, TAC CCATCGTCG CCTTGGT R, CGGACTAAAACCGCATACACTTG	*P. gingivalis* (Pg)	126 bp	(Suzuki et al., 2005)

aDue to uncertainty of taxonomic identification of *Act. viscosus* with respect to the identification of this species in human strains, we denote this species in quotation marks following Könönen (Könönen & Wade, 2015).

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 cm^2, (non-sintered, Hi-Med, Old Bethpage, NY) were placed into each well of a 12 well plate (Falcon, Corning, USA). Two millilitres of sterile of M-BHI was added to each well followed by 500 µl of the saliva/plaque inoculum and incubated at 37°C (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) 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 was used for DNA extraction. DNA was extracted using a boiling method (Khosravi et al., 2014), a simple and cheap method that has been shown to be effective for human dental plaque (Nance et al., 2013). The biofilm was boiled in dH_2O (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, 10 µM forward and reverse primers (Table S1) 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). One microlitre 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 s, 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). 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 dH_2O (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 h at 37°C to check for contamination. No evidence of growth by turbidity after 3 days of 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 the flow around the teeth we used neutrally buoyant (1.00 g/cm^3) blue polyethylene microspheres (180–212 µm diameter; Cospheric). The microspheres were first wetted properly by soaking in a hand soup solution for 2 h 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 an 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 $\gamma = u/d$. The shear stress at the tooth surface (τ_w) was calculated from $\tau_w = \mu\gamma$, where μ = absolute viscosity which we assumed was 0.75 cP (0.75 mPa.s or 7.5×10^{-4} Kg.m/s^2) based on that of a 0.9% saline solution at 37°C (Ozbek et al., 1977) to represent our media. Reynolds (Re) number was estimated from $\mathrm{Re} = \rho u L/\mu$ where ρ = density which we assumed was 1000 kg/m^3 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 τ_w 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) fitted with a bacterial vent filter (BST 02915 BioSurface Technologies) (Figure 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 3.1 mm (Cole-Parmer, Masterflex L/S16) with a peristaltic pump (Cole-Parmer, Ismatec IPC ISM932A) 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). 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

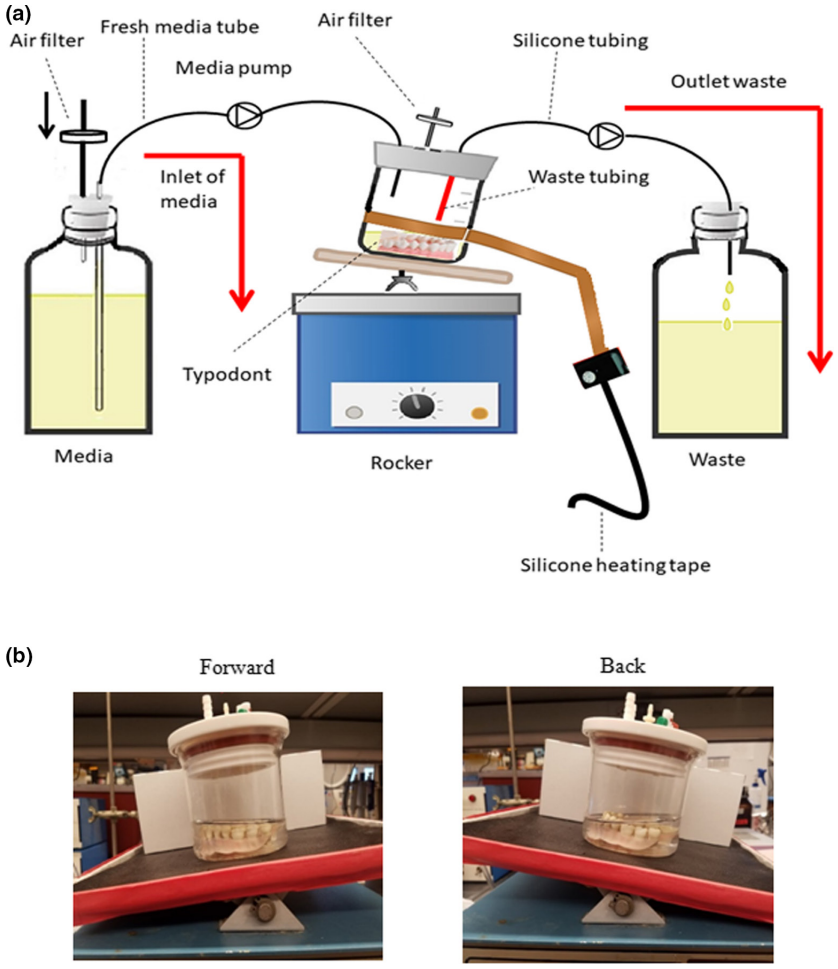

FIGURE 1 Dynamic typodont biofilm model (DTBM) (a) schematic showing the main components of the system. (b) Side view of the water level when the rocker was tilted all the way forward and then all the way back illustrating how the teeth were cyclically bathed in nutrient media.

table (Bellco, USA) which supplied a front to back rocking motion with an angle of 62°. The teeth in the mandibular jaw were numbered according to the "universal numbering system" (Figure S1). 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 into 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 min. The supernatant was then centrifuged (Legend micro 21; Thermoscientific, USA) at 10 G for 10 min. The supernatant was discarded, and the pellet was 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 Total^{SnF}) containing 0.454% (0.15% w/v fluoride ion) in the form of a 1:5 (w/v) solution by dissolving 1 g of toothpaste into 4 ml of dH_2O (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). 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). However, a dilution greater than 1:3 was shown to reduce efficacy (Satou et al., 2020). The final concentration of SnF_2 in our toothpaste solution was 0.0908% (908 μg/ml). After 24 h 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 h 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 h 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 hr and then staining with 0.05% MB for 30 s. 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). Briefly, the typodont was first rinsed with PBS to remove loose cells. Then, incubated with 150 ml MB for 30 s, 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) 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 cm^2 using estimates of supragingival tooth surface area (Kimura et al., 1977).

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 was used for DNA extraction using the boiling method (Khosravi et al., 2014). DNA concentration was calculated ng per tooth (DNA concentration × total volume of DNA) and also ng DNA per cm^2 using estimates of supragingival tooth surface area (Kimura et al., 1977).

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 variable factors for analysis. Statistical analysis was carried out based on two independent variables using a 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).

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 (Figures S2 and S3). Simulated plaque biofilms grown on HA coupons appeared patchy on day 1 but after 4 days of growth were more uniform and visibly thicker (Figure 2a,b). Simulated plaque biofilm on the typodont teeth was also patchy and for the molars appeared denser on the occlusal surfaces (Figure 2c). *Strep. oralis* showed a slight decrease in relative abundance after day 2 as the biofilm matured (Figure 3 and Figure S4). *P. gingivalis* and *Act. viscosus* were established in the biofilm after 1 day and showed an increasing trend in relative abundance as the biofilm matured. *V. parvula* and *Fusobacterium* spp. signals were saturated (>255 greyscale pixel intensity) for all or some of the replicates or days, thus relative changes over time for these bacteria were not possible (Figure S4). 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 (Figure S3). All targeted bacteria species were identified in the untreated SPB after 4 days of growth (Figure S5).

Biofilm treatment with the SnF_2 formulation

The typodonts stained with MB prior to removing individual teeth in the SnF_2 treated and control are shown in Figure 4. MB eluted from the individual teeth the molars and premolars had greater biofilm than the incisors and canines (Figure 5a). This was expected due to the larger surface area of these teeth and when normalized to tooth area the distribution is more even (Figure 5b). MB staining showed an average 53.0% reduction ($p < 0.0002$) in biofilm biomass

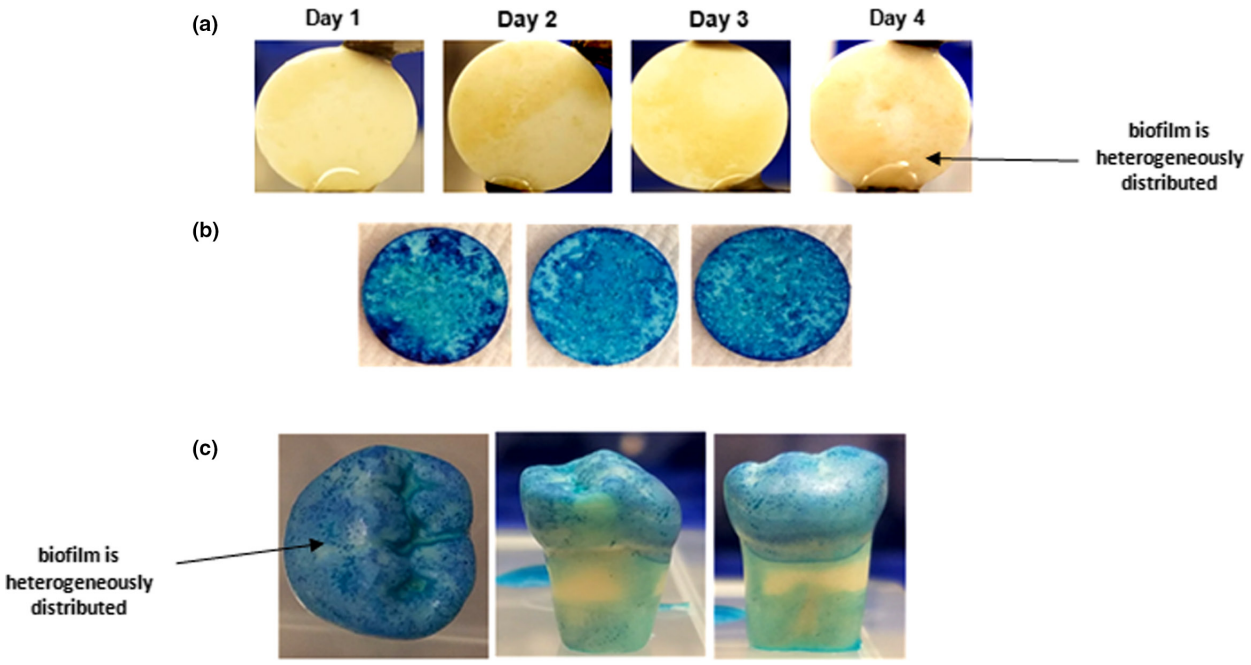

FIGURE 2 Progression of biofilm development on HA coupons. Over the 4 days of growth, the biofilm progressed in surface coverage on the coupon, becoming progressively more uniform by day 4. (a) Non-stained photographs of the simulated plaque biofilms on HA coupons after 1, 2, 3 and 4 days of growth.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

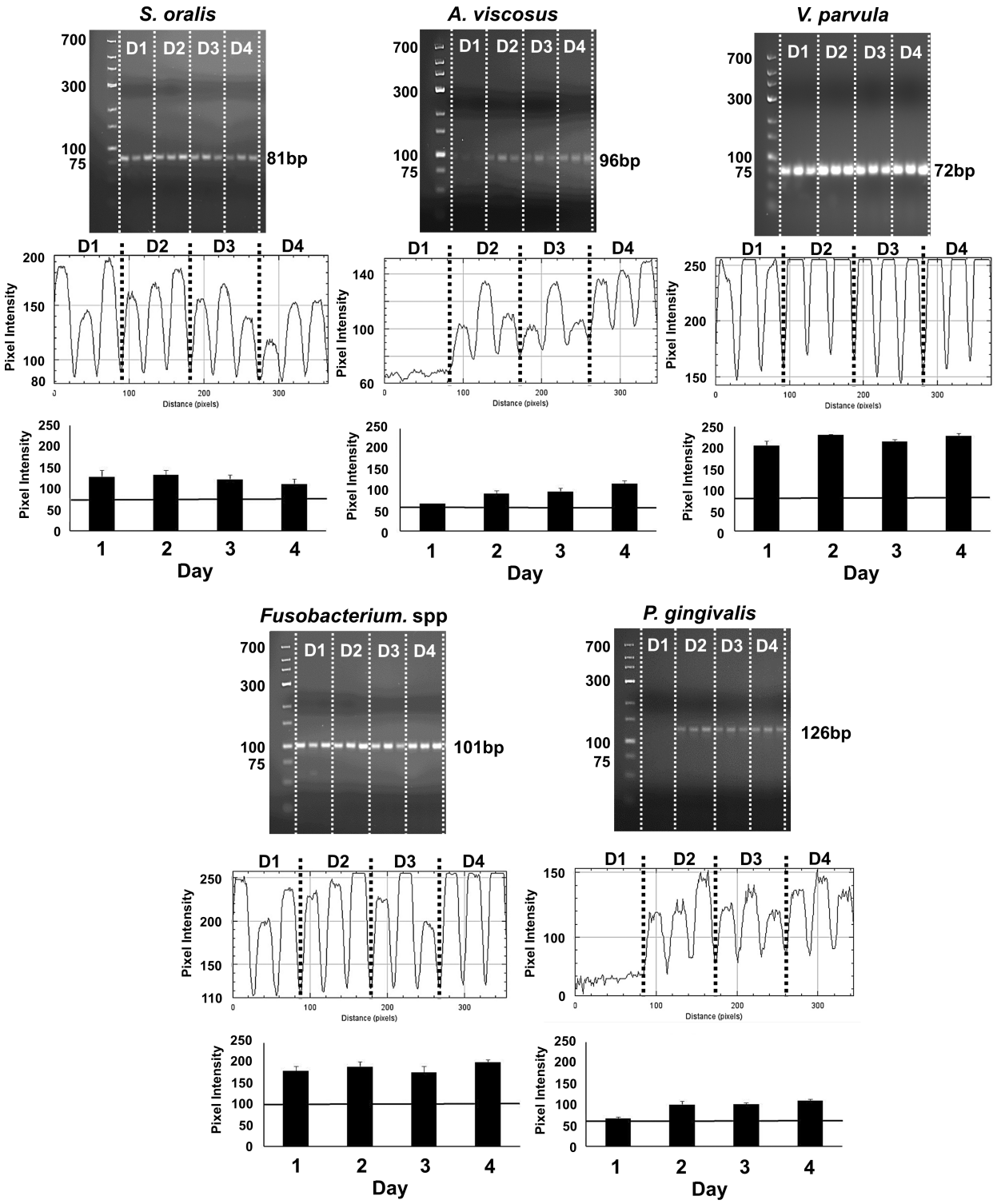

FIGURE 3 Representative image of densitometry data for the SPB over time. The original gels are shown above the pixel inensity from ImageJ "plot profile" and the graphed data of the pixel intensity is shown at the the bottom. Data are separted into each of 4 days for the three replicates by the dashed lines. Numbers to the left of the gels are the ladder bp. The band size of the PCR product for each primer set is indicated to the right of the gel. *Strep. oralis* showed a slight decrease from day 2, whereas *Act. 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 (Figure S4). The background grey level of the gel is indicated by the solid line on the pixel intensity graphs. Mean and 1 SD ($n = 3$).

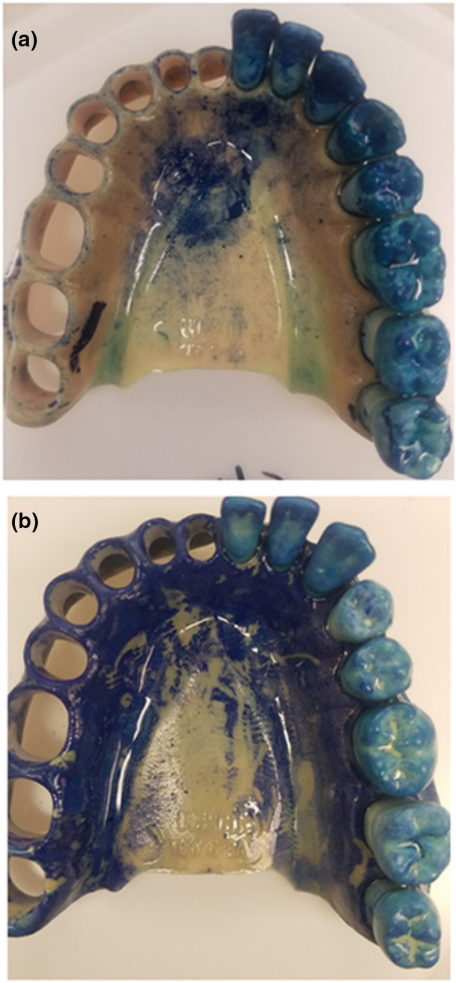

FIGURE 4 Typodont biofilm staining by MB. Simulated plaque biofilms grown on the typodont and then treated every 8 h for 4 days with (a) sham control rinse, (b) Colgate Total^{SnF}. On the last day, the typodont was removed and stained with MB (blue). The less intense staining of the SnF_2 treated typodont indicates less biofilm than the sham and was later quantified by elution from each individual tooth measurement by absorbance.

when treated with the SnF_2 solution typodont compared to the control ($p < 0.0003$) (Figure 5a,b). Total bacterial DNA showed a similar trend with greater biofilm on the molars and premolars (Figure 5c) with an average percentage reduction of biofilm of 54.4% ($p \leq 0.001$) (Figure 5c) calculated per cm^2. There was also a significant reduction of 53% for MB staining and a 54% reduction based on amount of DNA normalized for tooth area ($p < 0.0001$) (Figure 5d).

Estimate 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 (Movies S1–S4). 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 10–100 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 & Dawes, 1987) and fluid velocities have been estimated to range between 0.0013 and 0.0133 cm/s and an average shear stress at the tooth surface of "~0.8 dyn/cm^2" (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) 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; Sharma et al., 2005) but also the exchange of nutrients, dentifrices and metabolites between the biofilm and the overlying fluid (Stoodley et al., 2008). 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) 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

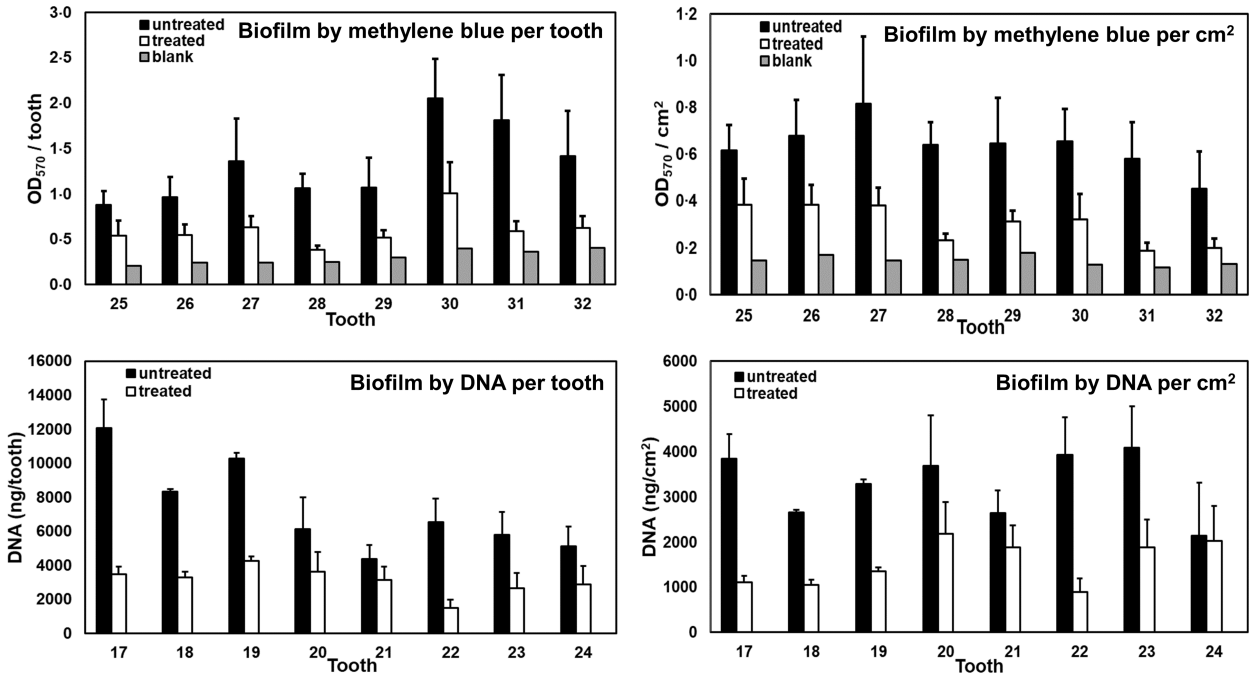

FIGURE 5 Treatment with SnF_2 formulation significantly reduces simulated biofilm plaque assessed by MB staining and amount of bacterial DNA per tooth. (a) MB staining showed that there was significantly less biofilm on the teeth treated with SnF_2 compared to the sham control $p < 0.0002$. The average 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 SnF_2 treatment compared to the sham control ($p < 0.001$). Similar to the MB staining there were greater amounts of DNA on the molars and premolars. (d) DNA amount per tooth normalized for tooth area shows a more even distribution.

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 *Strep. oralis* declined (Figure 3b). These trends are consistent with the development of human plaque biofilms which show a greater abundance of oral streptococci initially followed by increasing abundance of anaerobic pathogens (Teles et al., 2012) and is also consistent with our hypothesis that the consumption of oxygen by facultative species creates anoxic conditions favouring the 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 *Fusobacterium* 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 Total^{SF}-treated typodont compared to the sham control (untreated) ($p < 0.05$) (Figure 4a,b) and total bacterial DNA showed a similar average reduction of 54.4% total biomass ($p < 0.001$) (Figure 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 SnF_2 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 SnF_2 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). Repeated exposure to SnF_2 has been shown to significantly reduce the total amount of biofilm development in multiple in vitro reactor systems (Ledder & McBain, 2012; Ledder et al., 2010).

Although, we used a commercial SnF_2 formulation to validate that the typodont SPB model could produce an expected reduction in biofilm we did not assess whether this model had a greater predictive value that the simpler HA coupon model. The purpose of the work presented here was to assess whether the typodont model had the 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 (Figure 4) showed that biofilm was heterogeneously distributed on the typodont teeth. Interestingly, the fissures in the SnF_2 treated typodont visually had lower levels of staining than the untreated typodont, possibly reflecting that the biofilm in this area was less tolerant to SnF_2 or that SnF_2 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).

In conclusion, 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. Five species representing both early and late colonizers were 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 SnF_2 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.

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SUPPORTING INFORMATION

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