# Evidence of an In Vitro Coupled Diffusion Mechanism of Lesion Formation within Microcosm Dental Plaque

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## Declaration of Interests

None.

## Abstract

The purpose of this study was to determine whether or not the dual constant-depth film fermenter (dCDFF) is able to produce caries-like enamel lesions and to ascertain further information regarding the performance of this fully-functional biological caries model. Conditions were defined by the continuation (CF) or cessation (FF) of a saliva-type growth medium (STGM) supply during 50 mM sucrose exposures (8 times daily). Hydroxyapatite (n= 3) and bovine enamel (n = 3) substrata were included within each condition and samples extracted after 2, 4, 8 and 16 days. Community profiles were generated for fastidious anaerobes (FA), *Lactobacillus* spp., *Streptococcus* spp., Mutans streptococci (MS) and *Veillonella* spp. using selective culture techniques and enamel demineralisation assessed by transverse microradiography (TMR). Results demonstrated that the dCDFF model is able to produce caries-like enamel lesions with a high degree of sensitivity where reduced ionic strength (*I*) within the FF condition increased surface layer (SL) mineral deposition. Between conditions, biofilm communities did not differ significantly although MS in the biofilms extracted from the FF condition rose to a higher proportion (by 1.5 Log10 units) and *Veillonella* spp. were initially greater within the CF condition (by 2.5 Log10 units) indicating an enhanced ability for the clearance of low-pK­a acids following exposures to sucrose. However, both conditions retained the ability for caries-like lesion formation.

## Introduction

Dental caries results from complex interactions between oral biofilms, the enamel or dentine substratum, and host-specific factors (such as dietary intake of carbohydrates and hygiene) [Fejerskov and Manji, 1990]. Members of the biofilm community are able to ferment sugars and, as a by-product of this process, produce acids which dissolve the underlying hard tissue. If demineralising conditions offset intermediate periods of remineralisation, the net result is mineral loss and a carious lesion will develop. The interplay between these factors have been reviewed extensively and an ecological plaque hypothesis [Marsh and Bradshaw, 1997] has been extended to encompass current understanding of the process [Takahashi and Nyvad, 2008].

Although the microbial component is central to the disease process, research on non-biological models have led to far greater understanding of the mechanisms behind lesion progression [NIH, 2001]. The forefront of caries research therefore remains in investigating systems integrate physiochemical information within a biological context [Cochrane et al., 2010; Lynch, 2011; Rao and Maihotra, 2011; Robinson, 2011]; a feat which when applied *in situ* is generally confounded by a high degree of inter-individual variation [Larsen et al., 1999; Sullivan et al., 1995]. Exploring the effects of agents that modulate biofilm cariogenicity in vitro is therefore an attractive alternative and in this sense the Constant Depth Film Fermenter (CDFF) has emerged as a powerful tool to meet the needs of current *in vitro* research.

The CDFF has previously been used to model the growth of various microbial biofilms [Peters and Wimpenny, 1988] including the formation of dental plaque [Kinniment et al., 1996]. Essentially, there are three ecological modes of operation which are typically employed: single-species [Deng and ten Cate, 2004; Pratten et al., 1998b], defined multispecies [Kinniment et al., 1996; Shu et al., 2003], or microcosm multispecies communities [Hope and Wilson, 2003]. Whilst single-species biofilms allow experiments to be focused on a particular organism of interest, the lack of interaction between members of the wider microbial community limits their ability to mimic the clinical situation. Alternatively, defined communities provide a distinct advantage in that they can be used to replicate complex interactions within the oral ecosystem [Kolenbrander, 2011; Periasamy and Kolenbrander, 2009]*.* Ultimately, the microcosm biofilm model can be considered the most comprehensive since it represents the natural microflora in their entirety [Wimpenny, 1988]. In addition, the CDFF also affords an interface between solid, liquid and atmospheric media enabling a highly specific environment for biofilm growth when compared with microbial life in the planktonic state [Davies, 2003; Gilbert et al., 2002].

Biofilms grown in the CDFF have been used to model various response variables implicated in the caries process [Dibdin and Wimpenny, 1999; McLean et al., 2007; Pratten et al., 1998a; Wilson, 1999] including responses to fluoride and sucrose [Deng et al., 2005; Embleton et al., 1998]. However, those biofilms resulting from a microcosm inoculum have yet to be explored. Furthermore, whilst the CDFF has seen significant applications within caries research, carious lesions have yet to be produced within the model itself. Applications thus far have instead focused on oral biofilm production [Dibdin and Wimpenny, 1999; Hope et al., 2012; Kinniment et al., 1996], internal lesion formation on dentine [Cenci et al., 2009; Deng and ten Cate, 2004; Zaura et al., 2011], or *ex situ* enamel demineralisation [Aires et al., 2006].

Several theories which attempt to explain the architecture of natural caries lesions have also been put forward [Arends and Christoffersen, 1986]. *In situ*, the predominant cause of this characteristic has been attributed to the presence of fluorides during cyclic de- and re-mineralisation episodes. However, any mechanism which can exert some effect *in vitro* is likely to influence caries progression *in vivo*. To this end, direct evidence of the mechanisms which dictate carious lesion formation has yet to be provided through the use of biological caries model. The aim of the present study was therefore to demonstrate the ability of the CDFF model to produce caries-like lesions within bovine enamel tissue under exposure to sucrose as a cariogenic challenge. Additionally, the effect of varying operating conditions was explored by altering the supply of saliva-type growth medium (STGM) during sucrose exposures.

## Materials and Methods

### Inoculum Preparation

A saliva pool was created by collecting whole, unstimulated saliva samples from a random subset of healthy volunteers as described by Hope et al. [2012]. The saliva pool was split into a number of 1.8 mL aliquots and frozen at -80°C until required.

### CDFF Set-Up

For the purposes of this study, the dCDFF model [Hope et al., 2012] was adopted with some minor modifications (Figure 1). All liquid and silicone tubing was sterilised by autoclaving at 121°C for 15 min except sucrose-containing solutions, which were autoclaved at 116°C for 15 min. HA or enamel disks were placed into PTFE sample pans atop PTFE plugs and recessed to a depth of 200 µm thus providing a finite area for biofilm growth. Pans were then inserted into available spaces in the dCDFF turntable and the entire unit sterilised at 140°C for 4 h in a fan oven (Memmert UFB500; Memmert GmbH, Heilbronn, DE). Enamel disks were sourced from Modus Laboratories (Reading, UK) and stored in 0.1% w/w thymol solution before being sterilised external to the dCDFF units by gamma irradiation at 4080 Gy for 18 h [Amaechi et al., 1999]. Once sterile, the enamel samples were inserted into each dCDFF unit under laminar airflow.

### Media Preparation and Sucrose Exposures

An inoculation flask was prepared with 1 L of a mucin-containing saliva-type growth medium (STGM) [Pratten et al., 1998a] consisting of Lab-lemco 1 g.L-1 (Oxoid, Basingstoke, UK), yeast extract 2 g.L-1 (Oxoid), bacteriological peptone 5 g.L-1 (Oxoid), type III hog gastric mucin 2.5 g.L-1 (Sigma-Aldrich, Poole, UK), sodium chloride 0.2 g.L-1 (Sigma-Aldrich), potassium chloride 0.2 g.L-1 (Sigma-Aldrich), and calcium chloride di-hydrate 0.3 g L-1 (Thermo-Fisher Scientific, Geel, BE). Once cooled, the inoculum flask was located in an incubator at 37°C for 2 h before a single aliquot of the saliva pool was added. Whilst the contents were continuously stirred, peristaltic pumps (Watson Marlow, Falmouth, UK) were used to draw the inoculum though the tubing to enter each dCDFF unit at a flow rate of 0.5 mL.min-1. The inoculum was exhausted after approximately 16 h at which point a separate 10 L supply of STGM was pumped into each dCDFF unit by the same means but at a flow rate of 0.38 mL.min-1.

The sucrose pulsing regime described previously [Deng and ten Cate, 2004; Deng et al., 2005] was chosen as a template for this investigation with a revision to the procedure as illustrated in Figure 2. In brief, a 50 mM sucrose solution was pulsed into each CDFF unit at a rate of 0.38 mL.min-1, for 15 min x8 times daily over a 16 h period of a 24 h cycle. The study was continued for a period of 16 days. However, in one unit the supply of STGM remained continuous during exposures to the 50 mM sucrose solution (continuous-flow condition; CF) whereas in the other, the supply of STGM was halted at times when sucrose exposures occurred (feast-famine condition; FF).

### Analysis of the Salivary Type Growth Medium (STGM)

The native pH of the STGM was first measured from six 10 mL aliquots extracted from sterile STGM and the samples passed through a 0.2 µm Minisart syringe filter (Sigma-Aldrich) before being analysed by capillary electrophoresis (CE). Using the kits provided by the manufacturer (Beckman-Coulter Ltd., High Wycombe, UK), the concentration of chloride, sulphate, formate, succinate, acetate, lactate, phosphate, propionate, and butyrate (for anionic species) and ammonium, sodium, potassium, magnesium and calcium (for cationic species) was calculated relative to external standards set across 4 calibration levels. Lithium and azide were used as internal standards with sodium and chloride salts employed for all anionic and cationic separations respectively (Sigma-Aldrich). Fluoride was measured in each of the 6 filtrates using a calibrated (across concentrations in a predicted range of 1-1000µM of F standard from Sigma-Aldrich, r2 = 0.999) ion-selective electrode (ELIT 8221; Nico2000 Ltd., Middlesex, UK) with a 50:50 dilution in TISAB III buffer (Sigma-Aldrich).

### Recovery and Enumeration of Oral Bacteria

Following the extraction of each PTFE sample pan and immediately following the 4th sucrose exposure on the given sampling day, 3 of the HA disks supporting biofilms were removed and each placed in a sterile Bijou (Sterilin Ltd., Newport, UK) containing 5 mL PBS solution (Sigma-Aldrich) along with 3 sterile glass beads (3.5 - 4.5 mm diameter; BDH-Merk Ltd., Poole, UK) and vortex mixing for 30 sec. Serial 10-fold dilutions were then made in 1 mL PBS solution and 25 µL spread onto the following agars and incubated anaerobically for 48 h before determining viable counts by the number of colony forming units per unit area of supping biofilm growth (CFU.mm-2).

Viable counts of fastidious anaerobes (FA) were collected on fastidious anaerobic agar (Bioconnections, Leeds, UK) supplemented with 5 % (v/v) defibrinated horse blood (TCS Biosciences, Botolph Claydon, UK). Mutans streptococci were culture on a modified TYC medium (Lab M Ltd., Lancashire, UK) supplemented with 150 mg.L-1 sucrose (Sigma-Aldrich) and 3.5 mg.L-1of Bacitracin (Sigma-Aldrich) [van Palenstein Helderman et al., 1983]. Viable counts of *Streptococcus* spp. counts were obtained on MSA agar (BD Difco Co., Sparks, MD. USA) supplemented with 1 mL of 1 % (w/v) potassium tellurite solution (Sigma-Aldrich) and *Lactobacillus* spp. were enumerated using Rogosa agar (Oxoid).

*Veillonella* spp. were also quantified using an adaption of the method described by Rogosa [1956]. A base consisting of 15 g bacto agar (Oxoid), 5 g bacto peptone (Oxoid), 5 g yeast extract (Oxoid), 0.75 g sodium thioglycollate (Sigma-Aldrich), 2 mg basic fuchsin (Sigma-Aldrich) was weighed out and made up to a volume of 500 mL with dH2O. Twenty-one mL of 60% (v/v) sodium lactate (Sigma-Aldrich) was then added and the volume brought up to 1 L with further dH2O with the pH was adjusted to 7.5 with 1 M NaOH (Sigma-Aldrich) before autoclaving at 121°C for 15 min. Once cooled to approximately 48°C, 7.5 mg of filter sterilised vancomycin (Sigma-Aldrich) was added via filter sterilisation in 5 mL dH2O

### Enamel Lesion Quantification

Following the removal of biofilm, the enamel disks were subject to analysis by transverse microradiography (TMR). Thin (900 µm) sections were cut at an angle transverse to the exposed enamel surface using a precision diamond wire saw (Model 3241; Well Diamantdrahtsagen GmbH., Mannheim, Germany). Samples were then polished on both sides with a custom-made diamond-impregnated grinding disc (15 µm particles size; Buehler, Illinois, USA) to yield planar-parallel sections 80 µm in thickness.

Each thin section was then mounted on an acetate sample frame and placed film-side down on high-resolution x-ray film plates (Kodak type 1A High-Resolution Plates; Kodak, Rochester, USA) along with a 13-piece aluminium step-wedge and exposed to a CuKα X-ray source operating at 10 mA and 30 kV. Exposure time was 25 min and the distance from source to sample frame was 300 mm. X-ray sensitive films were developed in solutions provided by the manufacturer (Kodak D-19 Professional Developer; Kodak, Rochester, UK and Kodak Unifix; Kodak, Rochester, UK) following the instructions provided

Radiographic images were analysed using an optical microscope (Leica, Wetzlar, Germany) fitted with a CCD camera (Sony, Tokyo, Japan). Between 5 and 6 images capturing an area of 600µm acquired using the TMR 2000 software (Version 2.0.27.16; Inspektor Research Systems BV., Amsterdam) and parameters of integrated mineral loss (∆Z), lesion depth (LD), average mineral loss (R) and the degree of surface layer mineralization (SZMax) were measured using the TMR 2006 software package (Version 3.0.0.10; Inspektor Research Systems BV.).

### Statistical Analysis

Analysis of data was performed using SPSS Statistics 20 (Version 20.0.0.1; IBM UK Ltd., Portsmouth, UK). Results for viable counts were transformed (Log10) before statistical analysis and an Analysis of Variance (ANOVA) applied to all data collected. In the event that significant differences were found within groups containing more than 2 sets, Tukey’s HSD post-hoc test was applied. For all statistical tests, confidence intervals of 95 % certainty were applied (α = 0.05).

## Results

### Biofilm Composition between Substrata and dCDFF Conditions

Between dCDFF conditions, statistically significant differences were observed on each sampling occasion with respect to FA (P ≤ 0.014). FA reached a maximum on the 8th sample day in the FF condition although growth curves appeared non-district form those observed within the CF condition. Likewise, *Lactobacillus* spp. demonstrated remarkably similar trends between both dCDFF conditions. However, *Streptococcus* spp. did not show this same level of clarity. In this instance, a greater degree of variation was observed although no major differences were determined between either conditions (Table 2).

Viable counts of MS rose to a comparatively greater magnitude (by 1.5 Log10 units) within the FF condition similar to that seen for FA. Ultimately, both conditions exhibited a sharp increase in growth which terminated at similar values (6.25 ± 0.5 Log10 units). However, viable counts determined for *Veillonella* spp. were dissimilar between dCDFF conditions. In the CF condition viable counts were initially far greater (by 2.5 Log10 units) and proceeded to experience a decrease toward the end of the experiment. In the FF condition, this response appeared delayed, consistent with a late community bloom. For all microbial groups sampled, counts obtained from biofilms grown on either HA or enamel were also compared and no difference between the choice of either substrate could be found for the vast majority of possible combinations (P ≥ 0.053) with the expiation of 5 individual cases (P ≤ 0.026).

### STGM Ionic Composition

Analysis of the STGM determined absolute concentrations of each of the 12 analytes listed in Table 1. Formate and Ammonium were not detected in any of the samples measured. Likewise, fluoride was below the detection limits set by the analysis method (< 0.01 ppm). STGM pH was measured as 6.92 ± 0.07 SD therefore enabling the ionic strength (­*I*) to be calculated as *I* = 0.3118 mM [Shellis, 1988].

### Enamel Lesion Formation

In the FF condition a well-defined SL was evident in each of the samples analysed. However, in the CF condition, the SL was far less evident (Figure 3). Interestingly, lamination zones were apparent in some of the enamel sections taken from the latter condition (data not shown) as indicated by the large degree of variation observed in the samples extracted on the 8th sample day. Interestingly, adjacent enamel disks did not show this same variation in lesion character.

In comparing groups directly, it was not possible to accurately extract SZMax measurements on sample day 2 (due to the lack of any distinct SL structure) however parameters for ΔZ, LD and R were able to be analysed fully. Comparing these results found that, between dCDFF conditions, a significant difference was found with respect to ΔZ (P ≤ 0.013), LD (P ≤ 0.048) and R (P ≤ 0.009) on days 2, 4 and 16. However, on day 8, no significant difference (P ≥ 0.560) between any of these parameters were determined.

In the FF condition a progressive increase in ΔZ was seen over time (Figure 3). However, this same delineation could not be applied to CF condition. Here a significant increase in ΔZ was seen initially (P = 0.012) with no further increase following the 4th sample day (P ≥ 0.161). LD also demonstrated an extremely similar trend to ΔZ; measurements increased progressively over time and reached a maximum of 63.12 µm ± 4.90 µm SD by day 16 in the FF condition. Conversely, in the CF condition, LD was greatest in lesions removed on day 8. Trends in R showed some similarities to those for ΔZ and LD in that significant differences existed between dCDFF conditions at days 2, 4 and 16. However, when considering the data longitudinally, the trends were much less distinct. In the CF condition, no significant difference (P ≥ 0.146) between any of the time points sampled although in the FF conditions, some statistically significant differences were found to exist (Table 3).

As noted above, lesions created within the CF condition lacked obvious SLs but in the FF condition these areas were discernible on day 4, 8 and 16. Examination of the data collected found no difference between values for SZMax in the CF condition (P = 0.077). However a statistically significant difference was detected within measurements taken from the FF condition (P = 0.011).

## Discussion

In addressing the initial aim of this study, it can be concluded that the dCDFF model is able to produce caries-like enamel lesions under simulated sucrose exposures. Examining results further revealed that clear SLs occurred in lesions which were produced under the FF cycle whereas this feature appeared absent in the lesions taken from the CF condition. A possible basis for this observation may lie in chemical parameters at the interface between the enamel surface and biofilm PF. Coupled-diffusion has been identified as playing a significant role in the formation of the SL [Anderson and Elliott, 1987] and a factor which is able to augment this process is the ionic strength (*I*) of the medium at the interface [Anderson et al., 2004]. The combined introduction of STGM and the sucrose solution (ie. in CF condition) would, presumably, result in the biofilms being exposed to a medium of higher *I* than if they were exposed to sucrose in the absence of the STGM (FF condition). When mixed 50:50 within sucrose solution the bulk solution in contact with the biofilm during a cariogenic challenge would have a higher *I* in the CF condition than in the FF condition. It is therefore reasonable to assume that the PF would also be affected [Margolis and Moreno, 1994]. If the*I* of PF was significantly reduced in the FF condition then this would increase the effects of diffusive coupling within the system [Anderson et al., 2004] and therefore could explain the enhanced SL feature in lesions which were created under these conditions.

However, as a biological caries model, alterations to the collective physiological state of the biofilms within could be a factor that contributed to the lesions produced. Carbohydrates present in the STGM might not be high enough to result in significant levels of acid production to enable the selection of acidogenic species and ultimately create a cariogenic state. However, based on the assertion that adjunct sucrose is sufficient for this process [Aires et al., 2006; Arthur et al., 2013; Pratten et al., 2000], the reduction in sucrose concentration which occurs with the effect of mixing STGM and sucrose solution in the CF condition may have some influence on the lesions that are created. In effect, a 50 mM sucrose challenge in the FF condition would equate to approximately a 25 mM challenge in the CF conditions. Although a positive correlation was indicated, Aries et al. [2006] found no difference between these concentration ranges with their *in situ* model.

Bacterial enumeration showed only minor differences in viable counts between either of the dCDFF conditions. Such a degree of variation can be expected as a result of natural fluctuations known to occur within such multispecies consortia [Sissons, 1997; Skopek et al., 1993]. The lack of any real distinction between FA, *Lactobacillus* spp. and *Streptococcus* spp. demonstrates the efficacy of the dCDFF model. However, in the FF condition the proportion of MS rose higher whereas the proportion of *Veillonella* spp. remained lower. As highly acidogenic species [Marsh and Martin, 2009], a higher proportion of MS can be associated within a greater cariogenicity [Hamada and Slade, 1980; Loesche, 1986]. On the other hand, *Veillonella* spp. possess the ability to metabolise lactate as an energy source [Rogosa and Bishop, 1964]. Their presence within a biofilm may therefore aid in the removal of the acids following production [Mikx et al., 1976]. This would point to a less cariogenic plaque within the CF condition. However, assertions made on the basis of selective culture are limited as more than one community structure may be stable under a certain set of environmental conditions [Sissons, 1997]. In addition, any alteration to the environment is most likely to exert an effect on the biofilm phenotype whereas changes in the composition are likely to be secondary [Kinniment et al., 1996]. Consequently, quantification of the metabolic activity would provide a much greater degree of insight.

The lamination zones [Palamara et al., 1986] apparent in some of the samples taken from the CF condition may have resulted from exposures of purely chemical source [Damato et al., 1990; Lippert et al., 2012] but within dCDFF system, the environmental conditions are highly controlled [Pratten, 2005]. It is therefore more likely that these structures resulted from some deviation from the uninterrupted state of biofilm growth. Biofilms which form progressively would, in theory, react to a cariogenic challenge in a way dependent on their state of maturity. Lamination zones may also result from the successive formation of 2 SLs (the deeper of which being the remnants of an earlier lesion which penetrated further). In a logical progression, this histological feature may have formed through damage or removal of a mature cariogenic biofilm and the re-colonisation with less cariogenic growth. If such a disturbance was to occur *in vivo*, subsequent re-growth would require passage through the stages defined by the ecological plaque hypothesis [Marsh, 1994]. Alternatively continuous re-inoculation with a fully aciduric community would arise within the CDFF by the action of the scraper blades but re-establishment of the biofilm structure would still be expected to reduce cariogenic potential somewhat. Therefore, although the substrate would be re-inoculated with a cariogenic community, full cariogenicity would not immediately develop. It is therefore important to note the samples size used within the present work as this would have to potential to influence findings. Within the current design of the dCDFF model, the number of sample pans is limited which in turn limits the number of sampling occasions. In order to produce a data set reflective of both biofilm growth and enamel demineralisation through the period of operation, a compromise in sample number is unavoidable given the current design of dCDFF units.

Lesion progression in the FF condition showed the greatest development over time whereas the progression appeared to be hindered in the CF condition (Table 3). Heightened proportions of *Veillonella* spp. could have contributed to lower cariogenicity [Mikx et al., 1976] nevertheless variation was also seen in the lesions created under the CF conditions. To this end, the lamination zones could have contributed but their magnitude alone would not account for the observed degree of variation. If mechanical disturbance of the biofilm occurred at least once in the CF condition, then it is possible that this may have occurred several times. Depending on the extent of such a disruption, the biofilms may have been prevented from reaching their full cariogenic potential. Thus, mechanistic influences may have confounded results to some extent. However, a reduced cariogenic challenge resulting from mixing the sucrose solution with the STGM in the CF condition may also have contributed as was indicated by the higher proportion of MS and lower content of *Veillonella* spp. in the biofilms which received a greater exposure to fermentable carbohydrates.

Enamel lesion progression was also demonstrated to be non-linear within this model. These observations raise interesting questions over the dangers associated with stagnation sites and the removal of plaque biofilms as, from these data, cariogenicity was greatest in the earlier stages of the experiment; as biofilm maturation progressed, further demineralisation was limited (Table 3). This observation therefore questions the completeness of the extended ecological plaque hypothesis [Takahashi and Nyvad, 2008] as what would be observed is a biofilm which had reached a highly cariogenic state but following which a reversion had also occurred. Alterations in the structure and composition of the mature biofilm and the retention of inorganic mineral ions [Cury et al., 1997; Tenuta et al., 2006] could have contributed to the reduction in rate of mineral loss detected.

In conclusion, the CDFF model is able to produce caries-like enamel lesions internally. There is also supporting evidence that biofilms produced within the CDFF may be more highly cariogenic in the earlier stages of formation. Methods which are able to directly capture their metabolic activity should also be employed [Nyvad et al., 2013] in order to provide additional assessment of metabolic activity. Nevertheless, the nature of the dCDFF model was shown to be highly responsive with the degree of control available.

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## Legends

**Table 1:** Saliva-Type Growth Medium (STGM) composition as measured by CE. Formate and ammonium were not detected in any of the samples analysed (n = 6).

**Table 2:** Biofilm community profiles generated by selective culture for the microbial groups indicated (n = 6).

**Table 3:** Lesion parameters of integrated mineral loss (ΔZ; %Vol.µm), lesion depth (LD; µm), average mineral loss (R; %Vol) and SL mineralisation (SZMax; %Vol)

**Figure 1:** dCDFF Schematic Set-Up;a: Sterile Saliva-Type Growth Medium (STGM); b: Sterile 50 mM Sucrose Solution; c: Peristaltic Pump 0.38 mL.min-1; d: Peristaltic Pump 0.38mL.min-1 (CF); e: Peristaltic Pump 0.38 mL.min-1; f: Grow-Back Trap; g: Peristaltic Pump 0.5mL.min-1; h: Inoculum Flask with Magnetic Stirrer; i: CDFF Unit A; j: CDFF Unit B; k: Effluent; \*: 0.3 µm Air Filter. Note that “d” was responsible for the STGM supply to Unit A and therefore this pump functioned continuously whereas “e” functioned only at times when “c” pumps were inactive.

**Figure 2:** Saliva-Type Growth Medium (STGM) supply and sucrose pulsing strategy between dCDFF conditions; CF Condition = Continuous Flow Condition, FF Condition = Feast-Famine Condition.

**Figure 3 (Enamel Lesion Scan Profiles):** a) Lesions created within the CF condition. “Cont-Flow / Day 2”, “Cont-Flow / Day 4”, “Cont-Flow / Day 8” and “Cont-Flow / Day 16” conditions n = 27, n = 44, n = 37 and n = 37 respectively; b) Lesions created within the FF condition. “Fast-Famine / Day 2”, “Fast-Famine / Day 4”, “Fast-Famine / Day 8” and “Fast-Famine / Day 16” conditions n = 34, n = 32, n = 26 and n = 26 respectively. Error bars represent the sample SD.

## Tables

**Table 1**

|  |  |  |  |
| --- | --- | --- | --- |
| **Analyte** | **Concentration ± SD** | **Analyte** | **Concentration ± SD** |
| Chloride (mM) | 1.098 ± 1.154 | Propionate (µM) | 362.58 ± 99.17 |
| Sulphate (µM) | 417.49 ± 59.49 | Butyrate (µM) | 264.11 ± 246.47 |
| Succinate (µM) | 336.74 ± 88.23 | Sodium (mM) | 7.749 ± 0.903 |
| Acetate (µM) | 359.90 ± 31.17 | Potassium (mM) | 8.543 ± 0.616 |
| Lactate (mM) | 1.273 ± 0.382 | Magnesium (µM) | 277.40 ± 69.47 |
| Phosphate (mM) | 1.442 ± 0.320 | Calcium (mM) | 2.930 ± 0.463 |

**Table 2**

|  |  |  |
| --- | --- | --- |
| **Microbial Group** | **Sample Day** | **Viable Counts (Log10CFU.mm-2) ± SD** |
| **CF Condition** | **FF Condition** |
| FA | 2 | 8.15 ± 0.19 | 7.70 ± 0.14 |
| FA | 4 | 9.15 ± 0.18 | 8.36 ± 0.21 |
| FA | 8 | 9.54 ± 0.10 | 9.94 ± 0.21 |
| FA | 16 | 9.51 ± 0.17 | 9.24 ± 0.15 |
| *Lactobacillus* spp. | 2 | 4.62 ± 0.16 | 3.55 ± 0.18 |
| *Lactobacillus* spp. | 4 | 6.13 ± 0.06 | 6.26 ± 0.15 |
| *Lactobacillus* spp. | 8 | 6.76 ± 0.12 | 6.50 ± 0.11 |
| *Lactobacillus* spp. | 16 | 5.00 ± 0.23 | 5.86 ± 0.09 |
| *Streptococcus* spp. | 2 | 6.60 ± 0.40 | 6.44 ± 0.13 |
| *Streptococcus* spp. | 4 | 8.07 ± 0.11 | 7.26 ± 0.44 |
| *Streptococcus* spp. | 8 | 7.92 ± 0.14 | 8.20 ± 0.12 |
| *Streptococcus* spp. | 16 | 7.91 ± 0.18 | 7.45 ± 0.26 |
| MS | 2 | 5.17 ± 0.07 | 4.06 ± 0.43 |
| MS | 4 | 7.08 ± 0.06 | 6.23 ± 0.10 |
| MS | 8 | 6.69 ± 0.17 | 8.01 ± 0.14 |
| MS | 16 | 6.36 ± 0.11 | 6.04 ± 0.11 |
| *Veillonella* spp. | 2 | 4.82 ± 0.14 | 2.77 ± 1.36 |
| *Veillonella* spp. | 4 | 5.94 ± 0.11 | 3.51 ± 0.20 |
| *Veillonella* spp. | 8 | 6.22 ± 0.10 | 5.31 ± 0.18 |
| *Veillonella* spp. | 16 | 4.63 ± 0.21 | 5.33 ± 0.20 |

**Table 3**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Condition** | **Sample Day** | **n (Enamel Disks)** | **ΔZ ± SD** | **LD ± SD** | **R ± SD** | **SZMax ± SD** |
| CF | 2 | 3 | 282.23 ± 43.89 | 14.10 ± 2.02 | 19.70 ± 0.68 | 62.96 ± 2.88 |
| FF | 2 | 3 | 124.57 ± 45.75 | 7.96 ± 3.18 | 15.59 ± 1.32 | 71.39 ± 5.43 |
| CF | 4 | 3 | 483.75 ± 67.10 | 23.21 ± 3.40 | 21.14 ± 1.48 | 62.14 ± 2.97 |
| FF | 4 | 3 | 988.81 ± 138.22 | 34.92 ± 4.35 | 28.62 ± 1.48 | 51.42 ± 7.57 |
| CF | 8 | 3 | 1483.47 ± 1004.4 | 46.31 ± 21.96 | 28.14 ± 6.73 | 54.02 ± 5.37 |
| FF | 8 | 3 | 1602.00 ± 212.93 | 54.60 ± 5.37 | 28.20 ± 1.79 | 60.24 ± 6.24 |
| CF | 16 | 3 | 802.21 ± 91.40 | 38.86 ± 2.43 | 20.31 ± 3.39 | 62.99 ± 4.74 |
| FF | 16 | 3 | 2359.86 ± 291.96 | 63.12 ± 4.90 | 37.09 ± 2.87 | 50.63 ± 5.22 |

## Illustrations

**Figure 1**



**Figure 2**



**Figure 3**



## References

Aires CP, Tabchoury CPM, Del Bel Cury AA, Koo H, Cury JA: Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. Caries Res 2006;40:28-32.

Amaechi BT, Higham SM, Edgar WM: The use of gamma irradiation for the sterilization of enamel for intra-oral cariogenicity tests. J Oral Rehabil 1999;26:809-813.

Anderson P, Bollet-Quivogne FRG, Dowker SEP, Elliott JC: Demineralization in enamel and hydroxyapatite aggregates at increasing ionic strengths. Arch Oral Biol 2004;49:199-207.

Anderson P, Elliott JC: Coupled diffusion as basis for subsurface demineralization in dental caries . Caries Res 1987;21:522-525.

Arends J, Christoffersen J: Invited review article: The nature of early caries lesions in enamel. J Dent Res 1986;65:2-11.

Arthur RA, Waeiss RA, Hara AT, Lippert F, Eckert GJ, Zero DT: A defined-multispecies microbial model for studying enamel caries development. Caries Res 2013;47:318-324.

Cenci MS, Pereira-Cenci T, Cury JA, ten Cate JM: Relationship between gap size and dentine secondary caries formation assessed in a microcosm biofilm model. Caries Res 2009;43:97-102.

Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC: New approaches to enhanced remineralization of tooth enamel. J Dent Res 2010;89:1187-1197.

Cury JA, Rebello MAB, DelBelCury AA: In situ relationship between sucrose exposure and the composition of dental plaque. Caries Res 1997;31:356-360.

Damato FA, Strang R, Stephen KW: Effect of fluoride concentration on remineralization of carious enamel an in vitro ph-cycling study. Caries Res 1990;24:174-180.

Davies D: Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2003;2:114-122.

Deng DM, ten Cate JM: Demineralization of dentin by *Streptococcus mutans* biofilms grown in the constant depth film fermentor. Caries Res 2004;38:54-61.

Deng DM, van Maele C, ten Cate JM: Caries-preventive agents induce remineralization of dentin in a biofilm model. Caries Res 2005;39:216-223.

Dibdin G, Wimpenny J: Steady-state biofilm: Practical and theoretical models. Methods Enzymol 1999; 310: 296-322.

Embleton JV, Newman HN, Wilson M: Influence of growth mode and sucrose on susceptibility of streptococcus sanguis to amine fluorides and amine fluoride-inorganic fluoride combinations. Appl Environ Microbiol 1998;64:3503-3506.

Fejerskov O, Manji F: Risk assesement in dental caires; in Bader JD (ed): Risk assessments in dentistry. Chappel Hill, University of North Carolina Dental Ecology, 1990, p 215 - 217.

Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW: The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol. 2002; 46: 203-256.

Hamada S, Slade HD: Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Mol Biol Rev1980;44:331-384.

Hope CK, Bakht K, Burnside G, Martin GC, Burnett G, de Josselin de Jong E, Higham SM: Reducing the variability between constant-depth film fermenter experiments when modelling oral biofilm. J ApplMicrobiol 2012;113:601-608.

Hope CK, Wilson M: Effects of dynamic fluid activity from an electric toothbrush on in vitro oral biofilms. J Clin Periodontol 2003;30:624-629.

Kinniment SL, Wimpenny JWT, Adams D, Marsh PD: Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. Microbiol 1996;142:631-638.

Kolenbrander PE: Multispecies communities: Interspecies interactions influence growth on saliva as sole nutritional source. Int J Oral Sci 2011;3:34-54.

Larsen MJ, Jensen AF, Madsen DM, Pearce EIF: Individual variations of ph, buffer capacity, and concentrations of calcium and phosphate in unstimulated whole saliva. Arch Oral Biol 1999;44:111-117.

Lippert F, Butler A, Lynch RJM, Hara AT: Effect of fluoride, lesion baseline severity and mineral distribution on lesion progression. Caries Res 2012;46:23-30.

Loesche WJ: Role of streptococcus mutans in human dental decay. Microbiol Mol Biol Rev 1986;50:353-380.

Lynch RJM: Zinc in the mouth, its interactions with dental enamel and possible effects on caries; a review of the literature. Int Dent J 2011;61:46-54.

Margolis HC, Moreno EC: Composition and cariogenic potential of dental plaque fluid. Crit Rev Oral Biol Med 1994;5:1-25.

Marsh PD: Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994;8:263-271.

Marsh PD, Bradshaw DJ: Physiological approaches to the control of oral biofilms. Adv Dent Res 1997;11:176-185.

Marsh PD, Martin MV: The mouth as a microbial habitat; in Marsh PD, Martin MV (eds): Oral microbiology. Edinbourgh, UK., Elsiver Ltd., 2009, p 8 - 23.

McLean JS, Ona ON, Majors PD: Correlated biofilm imaging, transport and metabolism measurements via combined nuclear magnetic resonance and confocal microscopy. ISME J 2007;2:121-131.

Mikx FHM, Van der Hoeven JS, Walker GJ: Microbial symbiosis in dental plaque in gnotobiotic rats and in the chemostat; in Stiles HM, Loesche WJ, O'Brien TC (eds): Microbial aspects of dental caries: Proceedings of a workshop on microbial aspects of dental caries. Washington D.C., Information Retrieval Inc., 1976, vol 3, p 763 - 771.

NIH: Diagnosis and management of dental caries throughout life. NIH consensus statement 2001;18:1-23.

Nyvad B, Crielaard W, Mira A, Takahashi N, Beighton D: Dental caries from a molecular microbiological perspective. Caries Res 2013;47:89-102.

Palamara J, Phakey PP, Rachinger WA, Orams HJ: Laminated zones in carious human dental enamel. J Oral Pathol Med 1986;15:109-114.

Periasamy S, Kolenbrander PE: Mutualistic biofilm communities develop with porphyromonas gingivalis and initial, early, and late colonizers of enamel. J Bacteriol 2009;191:6804-6811.

Peters AC, Wimpenny JWT: A constant-depth laboratory model film fermentor. Biotechnol Bioeng 1988;32:263-270.

Pratten, Wills, Barnett, Wilson: In vitro studies of the effect of antiseptic-containing mouthwashes on the formation and viability of streptococcus sanguis biofilms. J Appl Microbiol 1998;84:1149-1155.

Pratten J: Growing oral biofilms in a constant depth film fermentor (cdff); in: Current protocols in microbiology. John Wiley & Sons, Inc., 2005. 6:B:1B.5:1B.5.1–1B.5.18.

Pratten J, Andrews CS, Craig DQM, Wilson M: Structural studies of microcosm dental plaques grown under different nutritional conditions. FEMS Microbiol Lett 2000;189:215-218.

Pratten J, Smith AW, Wilson M: Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. J Antimicrob Chemother 1998;42:453-459.

Rao A, Maihotra N: The role of remineralizing agents in dentistry: A review. Compendium of Continuing Education in Dentistry 2011;32:26-36.

Robinson C: Mass transfer of therapeutics through natural human plaque biofilms: A model for therapeutic delivery to pathological bacterial biofilms. Arch Oral Biol 2011;56:829-836.

Rogosa M: A selective medium for the isolation and enumeration of the veillonella from the oral cavity. J Bacteriol 1956; 72: 533–536.

Rogosa M, Bishop FS: The genus veillonella ii. : Nutritional studies. J Bacteriol 1964;87:574-580.

Shellis RP: A microcomputer program to evaluate the saturation of complex solutions with respect to biominerals. Computer applications in the biosciences : CABIOS 1988;4:373-379.

Shu M, Browngardt CM, Chen Y-YM, Burne RA: Role of urease enzymes in stability of a 10-species oral biofilm consortium cultivated in a constant-depth film fermenter. Infect Immun 2003;71:7188-7192.

Sissons CH: Artificial dental plaque biofilm model systems. Adv Dent Res 1997;11:110-126.

Skopek RJ, Liljemark WF, Bloomquist CG, Rudney JD: Dental plaque development on defined streptococcal surfaces. Oral Microbiol Immunol 1993;8:16-23.

Sullivan RJ, Fletcher R, Bachiman R, Penugonda B, LeGeros RZ: Intra-oral comparison and evaluation of the ability of fluoride dentifrices to promote the remineralization of caries-like lesions in dentin and enamel. J Clin Dent 1995;6:135-138.

Takahashi N, Nyvad B: Caries ecology revisited: Microbial dynamics and the caries process. Caries Res 2008;42:409-418.

Tenuta LMA, Del Bel Cury AA, Bortolin MC, Vogel GL, Cury JA: Ca, pi, and f in the fluid of biofilm formed under sucrose. J Dent Res 2006;85:834-838.

van Palenstein Helderman WH, Ijsseldijk M, Huis in 't Veld JHJ: A selective medium for the two major subgroups of the bacterium Streptococcus mutans isolated from human dental plaque and saliva. Arch Oral Biol 1983;28:599-603.

Wilson M: Use of constant depth film fermentor in studies of biofilms of oral bacteria. Methods Enzymol.1999;310: 264-279.

Wimpenny JWT: Introduction; in Wimpenny JWT (ed): CRC handbook of laboratory model systems for microbial ecosystems. Boca Raton,FL, CRC Press, 1988, p 1 - 17.

Zaura E, Buijs MJ, Hoogenkamp MA, Ciric L, Papetti A, Signoretto C, Stauder M, Lingstrom P, Pratten J, Spratt DA, Wilson M: The effects of fractions from shiitake mushroom on composition and cariogenicity of dental plaque microcosms in an in vitro caries model. J Biomed Biotechnol 2011;2011:135034. doi: 10.1155/2011/135034.