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Oral microbial biofilm models and their application to the testing of anticariogenic agents

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ABSTRACT

Objectives: This review paper evaluates the use of *in vitro* biofilm models for the testing of anticariogenic agents. *Data:* Caries is a biofilm-mediated oral disease and *in vitro* biofilm models have been widely utilised to assess how anticariogenic or antimicrobial agents affect the de/remineralisation process of caries. The use of enamel or dentine substrata has enabled the assessment of the relationship between bacterial activity and caries lesion initiation and progression and how this relationship could be affected by the agent under study. *Sources:* Only papers published in the English literature were reviewed. *Study selection:* Both 'open' and 'closed' biofilm systems utilising either single or multiple-species as defined or undefined inocula are analysed. *Conclusions:* There is a wide variety of *in vitro* biofilm models used in the assessment of anticariogenic agents. A reproducible model that mimics the shear forces present in the oral environment, and uses a defined multiple-species inocula on tooth substrates can provide valuable insight into the effectiveness of these agents. *Clinical relevance:* Biofilm models are important tools for the testing of the mechanism of action and efficacy of novel anticariogenic agents. Results from these experiments help facilitate the design of randomised, controlled clinical trials for testing of efficacy of the agents to provide essential scientific evidence for their clinical use.

Introduction

Dental caries is a common oral disease affecting both adults and children. It is a multifactorial disease brought about by the interplay of host factors, plaque bacteria and diet. Extensive efforts in controlling caries through increased public awareness, home and public fluoridation measures have led to a decline in the prevalence of caries in developed countries. Despite the decline in caries prevalence, it is still the most common childhood chronic disease in the United States, five times more common than asthma [1]. Furthermore, the majority of caries occur in a small segment of the public; generally from the lower socio-economic strata and education level or in those with disabilities [2]. It is also becoming increasingly frequent in the elderly as more individuals retain their teeth. In recent years, reports have emerged that the decline in caries incidence seems to have arrested and reversed [3, 4], motivating researchers to find new caries preventive strategies. The most widely used caries preventive agent is fluoride which mainly exerts its effect on the demineralisation-remineralisation balance occurring at the tooth-plaque interface. A greater understanding of plaque microbiota and its role in the caries disease has led to increased efforts in developing antimicrobial, antiplaque, prebiotic, probiotic, chemotherapeutic agents and other alternative strategies for caries control.

The current aetiology of caries is based on the Ecological Plaque Hypothesis, where the plaque ecological balance is considered to be the key factor in determining an individual's caries susceptibility [5]. Central to this is the role of dietary carbohydrates which are metabolised by plaque bacteria to produce acid end-products, resulting in a drop in environmental pH, which when prolonged below a critical pH, results in a net dissolution of minerals from the tooth structure. The relationship between plaque bacteria and tooth in disease is highly complex and does not follow the classic exogenous infection model. Koch's

criteria, where an individual pathogen is implicated in a specific disease, are inapplicable to the polymicrobial biofilm-mediated caries disease [6]. The bacteria associated with the caries disease have often been described as ‘opportunistic pathogens’; however it has been suggested that since the bacteria implicated are resident bacteria, they should be described as pathobionts and not pathogens [7, 8]. Oral micro-organisms form structured metabolically organised biofilm communities of interacting species that are spatially heterogeneous due to the various physico-chemical gradients developed within the communities of distinct oral ecological niches [9-11]. These biofilm communities change composition, structure and spatial distribution in dynamic response to environmental stress [12]. The properties of biofilm communities are more complex and extensive than the sum of the individual organisms involved [13].

Martin Alexander first used the term ‘microbial homeostasis’ in 1971 to describe the ability of the oral microbial community in health to maintain stability and integrity in a variable environment, despite the periodic occurrence of fluctuating pH during carbohydrate metabolism [14]. It implied that the composition of the biofilm was stable whereas in reality, the oral ecosystem experienced physiological changes which result in microbiological shifts [15-18]. Recently, Zaura and ten Cate [19] suggested that the term ‘allostasis’ better reflected the dynamism of these physiological changes occurring in the oral ecosystem, whereby allostasis was defined as ‘the process of achieving homeostasis or stability through physiological or behavioural change [20, 21].

The oral microbiome is highly diverse, with distinct characteristics amongst the microbial communities residing at different oral surfaces due to variations in local environmental conditions [22-25]. Recent culture-independent studies found more than 14 phyla in healthy subjects with a core oral microbiome shared amongst unrelated individuals, comprising of the

predominant species found in healthy oral conditions [26-29]. The predominant taxa belonged to Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria [29]. Differences in biofilm composition exist in health and disease [25, 30-32]. In caries, the microbial composition shifts towards disease (dysbiosis) where bacterial diversity decreases as disease severity increases [33]. Taxonomic characterisation however, is insufficient to assess the relationship between the microbiome and the disease state. Characterisation of the functional activities of the oral microbiome *in vivo* will give further insight into caries initiation and progression, facilitating the development of novel targeted anticariogenic agents [34].

Many culture-dependent studies had implicated *Streptococcus mutans* as the main bacterial aetiological agent in caries. However, the use of molecular and metagenomic methods revealed that *S. mutans* accounts for only 0.1% of plaque bacteria and 0.7-1.6% of bacteria in caries lesions [35, 36]. A recent metatranscriptomic study showed that *S. mutans* accounted for 0.73% of all bacterial cells in enamel caries lesions, 0.48% in open dentine caries lesions and 0.02% in hidden dentine caries lesions[37]. Other species such as the low-pH non-*S. mutans* streptococci, *Actinomyces* spp., *Atopobium* spp., and those from the genera *Veillonella, Lactobacillus, Bifidobacterium* and *Propionibacterium*, have been associated with the caries process [38]. A recent RNA-based study showed that caries lesions harboured a wide range of combinations of bacteria that varied greatly between individuals, between different lesion types and even between the same types of lesion [39]. In conclusion, caries therefore, is a microbiological shift whereby the acidogenic and aciduric species of the polymicrobial biofilm increase at the expense of acid-sensitive species.

Biofilms have been described as ‘functional consortia of microbial cells with extracellular polymer matrices that are associated with surfaces’ [40]. The biofilm mode of growth affects

their susceptibility to anti-bacterial agents, demonstrating as high as 1,000 fold increase in anti-bacterial resistance compared to their free-living planktonic counterparts [41, 42]. Older biofilms showed greater antimicrobial resistance compared to their younger counterparts[41, 43, 44] indicating that polymicrobial interactions amongst the biofilm community members and components of a mature biofilm can affect antimicrobial resistance [45-47]. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, conventionally used to evaluate the efficacy of antibiotics and antimicrobial agents, are carried out with the test agent in contact with the micro-organism for a prolonged period of time at a fixed concentration in artificial test conditions [48]. However, they do not replicate the clinical oral environment, where the chemotherapeutic agent is rapidly diluted by oral fluids and is retained at sub-MIC levels for a longer period. It is also not the intention to kill the plaque bacteria but to control or restore microbial homeostasis [15, 49, 50]. Hence, conventional methods such as the MIC and MBC to evaluate the effect of therapeutic agents against oral biofilm diseases are inappropriate.

Caries preventive agents work in a variety of ways; by slowing the demineralisation process or enhancing the remineralisation process. They can also exert their effect on the plaque ecology by interfering with the environmental pressures that upset the microbiological homeostasis into dysbiosis to produce a cariogenic environment[51]. For several decades, much of the caries preventive research was focused mainly on fluoride and chlorhexidine. With greater understanding on how plaque ecology influences the caries process, compounds containing essential oils [52, 53], metal ions [54, 55], plant extracts [56-59], phenols [60, 61], quartenary ammonium compounds [62], enzymes [63, 64], surfactants [60], xylitol [65, 66], calcium-based remineralising agents [67, 68], prebiotics [69], probiotics [69-72], nanohydroxyapatite[73], amelogenin-releasing hydrogels [74] and antimicrobial peptides [55, 75-77] have been explored. The use of photodynamic therapy [78-80] and a non-thermal

atmospheric plasma technique [81] as alternative antimicrobial strategies has also been explored. The preferred mode of action is not to kill the oral bacteria, but to maintain the beneficial bacteria at levels associated with health [13, 82]. Agents that exert a bacterial effect at sub-lethal levels and remain in the oral environment for a long period of time are thus preferred [83]. Simón-Soro and Mira (2015) recently postulated that due to the polymicrobial nature of the disease, antimicrobial treatments to treat caries would be unsatisfactory and preventive strategies should instead be directed towards modulating the microbial interactions involved and their functional output [39].

Ideally, biofilms, their internal interaction and interactions with external factors should be studied in their natural environment. This is difficult to do in the oral environment where the anatomical structures and tooth relationships provide several distinct eco-niches for plaque bacteria to reside. This complexity in bacterial relationship with the oral structures has led to the development of biofilm model systems to aid in our understanding of the microbiology of the oral microbiome in health and disease. These models vary widely in purpose, design and microbiological complexity; allowing detailed analysis of the component parts under controlled experimental conditions [84]. The importance of including biofilms in *in vitro* testing of novel caries preventive agents was highlighted by Zhang et al, (2015) who showed that the presence of a biofilm could influence the treatment outcome [73]. Experimental model designs evolved with increased understanding of the oral microbiome ecology and pharmacokinetics of the active agent; and the change in the clinical pattern of the disease and oral hygiene care due to lifestyle factors [85]. This present review provides a broad description of the various biofilm models commonly used in the study of caries preventive agents and how they have added to our understanding of the mechanism of action and efficacy of these agents.

Types of biofilm models commonly used in testing anticariogenic agents

A good *in vitro* biofilm model for testing caries preventive agents should have the following features: the biofilm under study should be representative of the natural diversity of the oral microbiome and characteristic of dental plaque; the growth medium representative of saliva and the pharmacokinetics of the agents to be tested should reflect that in the mouth [48]. It should be able to study the effects of the agents on bacterial metabolism and/or enamel demineralization.

Many studies on antimicrobials do not include enamel substratum in the study design as the emphasis is on the microbiological aspects. Without tooth substrates, such models are unable to study the interactions of bacterial metabolites with enamel/dentine structure or assess the relationship of bacterial activity to caries lesion formation and progression. Several *in vitro* biofilm models have been developed that vary widely in complexity and utility. Mono-cultures have been used to determine the physiological activities of specific bacteria species. Multiple species from defined inocula are used to study the interactions between bacterial species. The use of saliva or plaque-derived cultures to reflect more closely the natural diversity of the oral biofilm led to problems with characterisation and reproducibility of replicate biofilm samples [86, 87].

In general, *in vitro* biofilm models can be broadly categorised into ‘closed’ or ‘open’ systems depending on the nutrient availability (**Fig. 1**).

1. Closed system

    1.1. Agar plate

        This is the simplest biofilm model where bacterial growth on the agar plate resembles a biofilm consisting of bacterial cells embedded in an extracellular matrix. The agar

provides a solid nutrient for direct bacterial growth. This technique was later refined to allow growth of the bacterial biofilm on 0.45 µm cellulose nitrate membrane filters placed on the surface of the agar plates. This model has been used to determine the susceptibility of oral bacteria to light-activated chemicals [78, 88, 89].

1.2. Multi-well cell culture plate

Biofilms grown in multi-well cell culture plates provide the potential for high-throughput analyses. They can be grown under batch culture [90] in aerobic or anaerobic conditions; with and without mixing; either as monospecies, defined consortia [91, 92] or as plaque microcosms [63, 93]. The wells contain sterile growth medium and are inoculated with bacterial cells which adhere to the walls and bottom of the wells to form biofilms. Coupons placed in the wells can also act as biofilm substrata. Biofilm susceptibility to a test agent is carried out by adding varying concentrations of the test agent into the wells. Multi-well plates have been used to evaluate hydrolytic enzymes as possible plaque control agents [63] and the inhibition of *S. mutans* biofilm by naturally occurring compounds, apigenin and tt-farnesol [58].

Guggenheim and co-workers (2001) described the use of a 24-well plate to generate a supragingival plaque model (Zurich Biofilm Model) using a multi-species biofilm comprising of *Actinomyces naeslundii, Fusobacterium nucleatum, Streptococcus sobrinus, Streptococcus oralis* and *Veillonella dispar* for the study of plaque physiology and testing of antimicrobials [91]. However, a limitation of the Zurich biofilm model was that the biofilms were grown under anaerobic conditions with continuous carbohydrate exposure that were not reflective of *in vivo* conditions. A

variation of the multi-well plate, the Calgary Biofilm Device, provided rapid testing of various antimicrobial agents for bacterial eradication [94]. Removable pegs positioned on the upper lid of a 96-well plate were used as biofilm substrata and could be dismantled individually or collectively; with biofilm cell viability assessed using microscopic or plate counting techniques. One limitation of the Calgary Biofilm Model was the inability to vary the substratum material.

An improvement of this model, the Amsterdam Active Attachment (AAA) Model, was recently described whereby the upper lid of a 24-well plate was custom-fitted with clamps that could hold different types of substrata [43]. The AAA model allowed for high-throughput testing of multiple compounds at different concentrations with different treatment times within the same experiment. Despite lacking the features of a continuous flow model where shear forces from fluid flow could be generated to mimic saliva flow and pulsing of nutrients/agents was possible, the model was able to generate reproducible plaque-like biofilms and simulate the plaque pH changes that occur *in vivo* after carbohydrate consumption as observed by the production of the Stephan's curve generated by polymicrobial biofilms when exposed to sucrose [44]. This relatively simple model seemed to produce results with a high predictive value on the efficacy of a test agent [85]. However, the limitations of the multi-well cell culture plate model with the relatively small amount of biomass produced, its static environment of continuous exposure to sucrose and lack of metabolic clearance made it unsuitable for a multidisciplinary experimental design [95].

2. Open system

Open systems or flow displacement systems, allow for the simultaneous and continuous addition of growth medium and nutrients and waste product removal from the system[96]. Examples of flow displacement systems include the chemostat, constant depth film fermenter, flow cells and the artificial mouth biofilm model [97-100]. An open system model allows for bacteria to grow in a 'steady state' condition, whereby the rate of microbial growth is kept constant under constant experimental conditions [87, 101], with bacterial density, substrates and metabolic product concentrations maintained at constant levels with respect to the period of observation [102]. At this stage, biofilm cell accumulation plateaus as biofilm cell number doubling times reach their maximum [103]. Generally, all nutrients are supplied in excess except for one growth-limiting nutrient. The concept of 'steady state' assumes a stable balance of the component microbial species in a multi-species culture [104]. However, individual species have varying generation times and nutrient requirements, and reach a steady state at different times [105]. One approach is to consider the multi-species culture as a single unit of activity in the analysis [87].

2.1. Biofilms grown in chemostats

A chemostat is a bioreactor where the influent (fresh medium) flow rate is equal to effluent (metabolic end products, microorganisms and left-over nutrients) flow rate, thus maintaining a constant culture volume [106]. However, conventional chemostats, being planktonic systems, are not representative of biofilm communities. To simulate the oral environment, the chemostat model system was improved whereby suspended substrate coupons provided solid surfaces for biofilm formation, allowing for microbial colonisation with spatial heterogeneity. Many different types of substrates have been used with the chemostat system and these can be removed at

different time points during the experiment for analysis or be transferred to another chemostat with a different environmental condition for further testing [100, 103, 104, 107, 108]. The inclusion of a solid substrate for biofilm formation poses an issue as to whether the system operated under steady state conditions as biofilms grown on solid substrata can form increasing attached biomass [109]. Increased demand for nutrients further upsets the steady state balance. In reality, microbial biofilms are complex bacterial communities with spatial heterogeneity and pH gradients across the depth of the biofilm. Access to nutrients is more limited for bacteria residing in the depths of the biofilm than those that are more superficially located. Hence, cell growth may not be constant throughout the biofilm structure.

2.2. Flow cells **(Fig. 2**)

The flow cell biofilm model consists of a liquid growth medium reservoir attached to single or multiple transparent chambers of fixed depth. Inoculation is carried out by passing a culture through the flow cell first to facilitate bacterial cell adherence before passing the growth medium through it. Both single-and multi-species biofilms can be produced; and different substrata can be tested within the same experiment. Flow cell orientation affects biofilm thickness; horizontally placed flow cells produce more plaque compared to vertically placed ones [110]. It allows for non-destructive real-time microscopic examination of the biofilm as it can be mounted on a microscopic stage[111]. However, its response to anticariogenic agents can only be determined using confocal laser scanning microscopy, whilst other means of assessments require disassembly of the apparatus. Real-time comparisons of multiple biofilms require multi-channel systems to construct the replicate biofilms for side-by-side comparisons.

Flow cells have been used to evaluate the effect of 0.03% triclosan mouthrinse on biofilm formation on hydroxyapatite and germanium compared to placebo controls [110]. The triclosan group showed significant reduction in optical density of the bacterial plaque formed on hydroxyapatite surfaces and plaque protein content on germanium surfaces. Another study reported the inhibitory effect of tin (IV) fluoride compound on *S. sanguinis* adhesion on glass substrates, which was dependent on pre-treatment formation of the conditioning film [112]. Using a defined mixed-species inoculum, Lynch and ten Cate (2006) reported the dose-response relationship of calcium glycerophosphate remineralising agent with regards to reducing tooth mineral loss and the importance of timing of delivery of the agent with respect to carbohydrate intake [113].

2.3. Constant depth film fermenter

To study the cause and effect relationship, time as a variable has to be removed as when a biofilm first develops, the community composition and proportion changes until it becomes stable (steady-state), following which the effect of any perturbation can then be easily quantified. A steady-state biofilm can be developed by allowing it to grow to a pre-determined depth after which the surface growth is continually removed to maintain a constant geometry; an approach first described by Atkinson and Fowler [114] (1974) and developed by Coombe et al (1981, 1984) using the constant depth film fermenter (CDFF) [115, 116].

The CDFF (**Fig. 3**) consists of a glass container with several ports for the entry of gas, inoculum and medium and access port for aseptic removal of samples at different time points during the experiment. Within the container sits a rotating turntable which holds 15 polytetrafluoroethylene (PTFE) sampling pans rotating

under two PTFE scrapers at defined speeds. Each sampling pan has five circular wells containing PTFE pegs recessed at pre-determined depths to create space for biofilms to form. Different types of substrates can be used within the same experiment.

The CDFF offers several advantages over other models: a large number of replicate biofilms can be produced and the resultant environment is similar to that of the oral cavity as the growth medium flows over the substrata in thin films, mimicking saliva flow [98, 111]. The movement of scraper blades mimics tongue movement and chewing forces. Reproducibility is achieved as the biofilms are grown at a constant depth and the reproducibility within a run has been shown to be good[98]. However, significant bacterial composition variations were observed between runs when mixed species biofilms derived from defined or saliva-derived inocula were used, affecting interpretation of results [105, 117]. This could be due to the heterogeneity of the inocula which became magnified by the growth conditions of the medium culture and the biofilm formative phases in the fermenter [118]; and the presence of unculturable species in saliva-derived inocula [119]. Attempts to address this issue included the use of a split design where the CDFF was divided into two independent sides, each with its own scraper bar and delivery ports [120]. Instead of rotating 360^0, the turntable could only oscillate over 180^0 and was only able to hold fewer sampling pans, restricting the number of biofilm samples that could be produced. No cross-over contamination of bacteria or test agents between the two sides was observed. The advantage was that two treatment groups could be tested within the same experiment. Experimental variability could also be minimised by operating two CDFFs concurrently instead of in series, whereby both CDFFs were concurrently

supplied by the same inoculation culture and artificial saliva growth medium using dual-channel pumps [121]. Another problem that CDFF users faced was the possibility that the biofilms might not grow to fill up the recessed space. Though the bacteria in these biofilms could likely settle into a 'steady state' level, they were less reproducible compared to those that filled the recessed depths completely [122].

2.4. Artificial mouth

An artificial mouth is an attempt to simulate the oral microbial environment *in vivo* under defined controlled experimental conditions. A central characteristic of this model is the growth of plaque bacterial microorganisms as biofilms on surfaces irrigated with nutrient and saliva. The artificial mouth is thus a laboratory microcosm, replicating many physical aspects of the oral cavity [109]. Many artificial mouth studies use saliva-derived inocula to produce plaque microcosms as this model has been shown to closely replicate the heterogeneity and variability of oral biofilms present *in vivo* [95, 118, 123, 124]. However, comparison of results derived from different saliva inocula is difficult as marked inter-individual variations in salivary species abundance [118] can lead to differing microbial responses to a test agent [123]. Such inter-individual variation can be minimised by the use of an inoculum derived by averaging or pooling the saliva from several individuals [125].

Factors to consider in biofilm model design

1. Study objective

The biofilm model selected depended on the study objectives and how close it should be to clinical reality. Steady state biofilm models where true cause-and-effect relationship can be established through independent adjustment of experimental parameters are preferred for the testing of antimicrobial and chemotherapeutic agents. An experiment

requiring a large number of sample replicates for sampling at multiple time points would likely favour the use of a CDFF. Another factor to consider is study duration; multi-well plates are unsuitable for use in a lengthy experiment due to limited nutrient availability.

2. Inoculum

Mono-species biofilm models are not representative of the clinical environment. In reality, oral bacterial species do not live independently but co-aggregate to form multi-species communities with synergistic co-operation in metabolic activities and growth [10, 11]. Over 700 species of bacteria reside in oral communities forming distinct ecological niches [126]. Co-cultures of two or three species allowed for the study and interpretation of the bacterial interactions involved. However, extrapolation of the findings from these reductionist models to the natural oral environment should be viewed with caution as the bacterial interactions and metabolic co-operation *in vivo* are complex and extensive [10, 127, 128]. To simulate the natural environment, several researchers have used saliva or plaque as the inoculum [103, 107, 129-131].

The advantage of using an inoculum derived from natural sources is that full representation and natural diversity of the oral microbiome are included in the experiment. The disadvantages of using such inocula, however, are numerous. The site and subject-specific diversity of the inocula make analysis and quantification of the composition of the resulting microbial communities extremely difficult. Certain bacterial species that are critical to the experiment may not be present in the inoculum; instead other undesirable species can affect the experimental outcome. Other problems include the inability to manipulate the inoculum composition for specific experimental objectives and issues involved in standardising the inoculum composition to obtain replicate plaque samples [86, 87].

Inocula with defined composition have been proposed to overcome the problems encountered with the use of saliva or plaque-derived inocula. The composition of the defined inocula can be constructed according to their relevance to the study and ease of identification and is useful for modelling the oral biofilms [105, 132]. The communities that developed are reproducible and stable with time. With defined multi-species consortia, one can determine the direct and indirect effect of a test agent on the various bacterial species, whereby an inhibitory effect on one species may result in an indirect effect on another species. It also allows for the study of the complex interdependent interactions such as metabolic co-operation [133] and bacterial co-aggregation [127, 134]. A defined inoculum consisting of ten bacterial species was shown to be able to produce reproducible steady-state microbial communities with respect to bacterial composition and metabolic activities [100].

A point to note is that *in vitro* laboratory reference strains used often in *in vitro* biofilm studies might not be representative of the bacterial species present in the *in vivo* environment. Genomic differences between laboratory reference strains and corresponding clinical strains have been reported, whereby the clinical strains showed greater genomic plasticity and pathogenicity [135, 136]. Standard laboratory growth conditions would not be able to reproduce the adaptive processes that occur *in vivo*, resulting in the loss of important patho-physiological characteristics involved in biofilm formation and virulence in the reference strains. The use of species-specific 'communal gene-pools' assembled in a virtual supragenome that reflects natural population variation could be employed in future biofilm studies [137].

3. Growth medium

Compositional variations in growth medium can affect the composition of the biofilm formed. *In vivo*, oral bacteria rely mainly on host-derived proteins and glycoproteins for their nutritional needs, with dietary carbohydrates a source of energy for the acidogenic species. To mimic the oral environment, growth media thus contained proteins and glycoproteins, with hog gastric mucin as the main energy and carbon sources [87, 104]. The use of proteins and peptides in the growth medium supported the growth of a diverse microbial community as the catabolism of complex proteins required synergistic glycosidase and protease activities amongst the different species [133]. Hog gastric mucin has the highest similarity in oligosaccharide structure with human salivary mucin [138]. Metabolism of the oligosaccharide side-chains of the mucin provided carbohydrates for bacterial growth [129]. Free sugar content was minimal (<0.05% w/v), allowing for the addition of pulsing of sucrose to simulate dietary carbohydrate [139]. Defined multi-species biofilm grown in a glucose-limited medium showed different compositional proportions compared to one grown in a glucose-excess medium [103]. Feeding regimes have also been shown to alter the resultant community composition of salivary derived microcosms grown in a CDFF [140]; the biofilms exposed to feast-famine regimes (artificial saliva supplemented with periodic feeding of complex nutrients), showed greater proportion of Gram negative anaerobes, Lactobacilli and *S. mutans* than those exposed to only artificial saliva medium.

4. Dilution rate

The dilution rate is defined as the ratio of the medium flow rate to the culture volume in the reactor and is described as the number of complete volume-changes/hour [101]. The dilution rate is controlled by the delivery rate of the medium into the reactor and determines the residence-time of the bacterial cells within the reactor. Bacterial cells form

biofilms when the dilution rate is less than the mean generation time of the bacterial species under investigation. Otherwise, the bacterial planktonic cells get washed away when the dilution rate exceeds their mean generation time. The biofilm continues to grow until it reaches a steady state. In reality, thicker or older biofilms will have increased spatial heterogeneity and access to nutrients is determined by the relative position of these cells in the biofilm structure, resulting in differential cell growth and physiological heterogeneity of the cells. The flow characteristic, whether it is laminar or turbulent, also influences the biofilm structure. As shown in a study on a biofilm model of mixed species of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas fuorescens* and *Stenotrophomonas maltophilia*, grown in glass flow cells, the biofilms grown in turbulent flow formed elongated micro-colony structures along the downstream flow; whereas biofilms grown in laminar flow formed circular-shaped micro-colonies interspersed with water channels [141].

5. Biofilm age

Biofilm age relates to the time of accumulation of biofilm [142]. In a semi-defined medium at a dilution rate of 0.1/h and pH 7, growth rate of a mixed culture comprising different *Streptococcus, Actinomyces* and *Lactobacillus* strains, reached a plateau after 24 h and the biofilm was composed of several layers of cells [103]. Longer accumulation of the biofilm will result in increased thickness and spatial heterogeneity; the physiologically heterogenous cells are not in steady state. Older biofilms show increased antimicrobial resistance and can influence the bacterial reactions involved in alkali production [43, 44]. They are also more resistant to acid pH [143, 144] and fluorides [43, 108] than younger biofilms. Old biofilms are representative of plaque found in oral stagnation sites which may be thicker and have different composition to plaque located at

easily accessible sites [145]. Studies using different ages of biofilms are useful to determine their relative responses to inhibitors.

6. Shear forces

Biofilms grown *in vivo* are exposed to shear forces contributed by saliva flow rate and direction. The forces vary depending on the location and anatomical variation of oral structures. Ideally, such forces should be replicated in *in vitro* biofilm models. Higher shear stresses produced more rigid and homogenous biofilms [146]. Open systems utilise fluid flow (chemostats, flow cells) or scraper blades (constant depth film fermenter) to create continuous detachment forces. Shear forces can be created in batch culture models by subjecting the biofilms in multi-well plates to gentle swirling or dip-washing, exposing them to passage through an air-liquid interface [91, 147].

7. Atmosphere

Supragingival plaque is comprised mainly of obligate anaerobic species with preferential growth in carbon dioxide (CO_2) enriched conditions. Mixed cultures comprising of facultative and obligate anaerobes grown in oxygenated conditions (gas phase of 5% CO_2 in air) showed an increasing proportion of obligate anaerobes with time, becoming predominant after 4-7 days [148]. This suggested that mixed cultures could protect obligate anaerobes from the toxic effects of oxygen enabling their growth in aerated conditions, a theory validated when a community comprising only four obligatory anaerobes did not survive when subjected to oxygen stress [149]. Hence, obligate anaerobes leveraged on the presence of aerobes/facultative anaerobes within the biofilm for survival. Biofilm studies that are carried out under anaerobic conditions usually operate under gas conditions of 5% (v/v) CO_2 in nitrogen.

8. Timing of delivery of test agents

The importance of timing of application in relation to efficacy of the chemotherapeutic agent is often overlooked. For example, should one use an antimicrobial or remineralising mouthrinse before or after a meal? This issue was elegantly highlighted by Lynch and ten Cate where they showed in a defined multi-species flow cell model, that calcium glycerophosphate, an organic calcium phosphate salt, was more effective in reducing enamel demineralisation when pulsed before the addition of sucrose than during or after the sucrose pulses [113]. The clinical implication suggested that the use of calcium glycerophosphate remineralising agent before dietary intake might have a greater anti-caries effect than using it after a meal. Timing of fluoride application however, had no effect on reducing the enamel demineralisation produced in an *in vitro* pH-cycling study [150].

9. Substratum

The type of substrate used in the experiment would depend on the study objective as it could influence the experimental results. Substrate materials vary to include glass, PTFE, polystyrene, polyacrylate, germanium, restorative materials, dental tissues and their analogs [43, 91, 103, 105, 107, 108, 110, 113, 143, 151-154]. The nature of the substratum surface affects the metabolic activities of the biofilms and their antimicrobial susceptibility [143, 155], depending on their differential ability to bind to the different substrates, their substantivity and their effectiveness in inhibiting bacterial adherence and cell viability. When exposed to sucrose challenge, *S. mutans* biofilm grown on polyacrylate substratum showed greater pH drop compared to dentine substratum and significantly lower counts when exposed to 0.2% chlorhexidine [143]. Triclosan was

more effective in inhibiting biofilm formation on hydroxyapatite surfaces than bovine enamel surfaces [155].

Antimicrobial agents can also be incorporated into the substrates and the effect of their release from the substratum on bacterial cell viability measured [108]. Substrate selection and surface characteristics affect adsorption of molecules and bacterial cells, biofilm cell numbers and metabolic activities. In a three-species biofilm model, fluoride released from hydroxyapatite rods was shown to affect fluoride-sensitive species [108]. The surface energy of substrates can influence the characteristics of the conditioning film that developed [87] and the amount of early bacteria deposits but not the structure of the biofilms grown on them [110, 156]. Salivary pellicle coating of different substrates can modify their surface properties [144, 157]. Hence, care must be taken when extrapolating antimicrobial efficacy results from *in vitro* biofilm studies to the clinical setting. Bovine enamel is often used as an alternative to human enamel but its relatively greater porosity and faster rate of demineralisation has to be taken into account when interpreting the results [158].

10. Outcome measures and analysis

Models which use tooth structure as substrates allow for the assessment of the relationship of bacterial activity to caries lesion initiation and progression and how this relationship can be affected by the anti-caries agent under study. The efficacy of a test agent can be determined by different outcome parameters, relating the cariogenic potential of the biofilm to its influence on tooth substrata. The most relevant outcome parameter is mineral quantification. Other useful parameters include reduction of acid

producing potential of biofilms and microbial shifts towards health-associated bacteria species [59].

The microbiological effects can be determined by measuring the changes in microbial composition [61, 100, 148], biomass or bacterial protein [58, 59, 159, 160], biofilm acidogenicity [44, 58, 143], polysaccharide production [58, 160], production of bacterial metabolites such as organic acids [43, 59, 73, 120, 143] and ammonia[44], cell viability [43, 58, 80, 143, 161, 162] and biofilm mineral content [73]. Microbial composition and cell viability can be characterised using differential culture techniques [43, 63], confocal laser scanning microscopy [63, 100, 163, 164] (**Fig. 4**), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) [63, 165, 166], fluorescence microscopy [63] and 16S rRNA sequencing [95]. Cryosectioning can be done to assess the distribution of various bacteria species throughout the biofilm [117]. The novel use of green fluorescent protein as a metabolic activity indicator in *S. mutans* biofilms [151] has the advantage of real-time fluorescent signal detection without disturbing the biofilm structure and cells. The selection of assessment method will also depend on the type of substrate used in the experiment; for example, bacterial growth on germanium surfaces is analysed using attenuated total reflectance Fourier transform infra-red spectroscopy [110].

Effects on tooth tissues can be determined by the surface microhardness [74, 159, 167-169], change in mineral content and depth of lesions [73, 113, 120, 152] in enamel and dentine. Knoop and Vickers surface microhardness tests are limited in their ability to assess the mineral status of more demineralised lesions [158]. Mineral ion content in enamel can be measured using multi-elemental analysis [168] and mineral content change evaluated using several methods such as confocal laser scanning microscopy [170],

transverse microradiography [73, 113, 120, 152, 163] and quantitative light fluorescence [147]. An advantage of using transverse microradiography (**Fig. 5**) is the ability to determine lesion depth inhibition by the test agents [120]. Baseline integrated mineral loss (ΔZ_{base}) levels in artificial caries lesions can affect their subsequent biomineralisation behaviour [171, 172]. When artificially demineralised lesions are subjected to further demineralisation, smaller lesions with lower ΔZ_{base} values show greater demineralisation compared to larger lesions with higher ΔZ_{base} values. This is attributed to the reduced solubility of the larger lesions due to the loss of carbonate and magnesium ions. However, the reverse is observed for the remineralisation process whereby the extent of mineral gain is directly proportional to the size of the lesion; likely due to the porosity of the lesions allowing diffusion of ions into the lesion. Hence, studies involving the use of artificial caries lesions should indicate the ΔZ_{base} values and their variability. Mineral gain/loss should be expressed both as percentage change of ΔZ_{base} value and as absolute difference in mineral content.

Conclusion

An effective and efficient *in vitro* biofilm model experimental design for the study of caries preventive agents has to take into consideration the study objectives, pharmacokinetics of the active agent, appropriate substrate selection, inoculum and growth medium and suitable outcome measurements. More than one outcome variable should be assessed to cross-check the validity of the results. Identification of novel promising compounds and improved assessment strategies to evaluate efficacy of these agents are needed to help identify novel agents, expand our understanding of their mechanism of actions and determine their possible effective concentrations and mode of application. This will help facilitate the design of *in situ* and *in vivo* experiments for testing of their clinical efficacy.

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Figure Legends

Figure 1. Conceptual differences between closed and open systems. (i) Closed system with batch culture: depletion of nutrients with time limits the duration of the experiment. (ii) Open system enables the simultaneous and continuous addition of nutrients and growth medium and removal of waste product. When the system reaches a steady state as observed in a constant depth film fermenter, the biomass remains constant (a). Biomass increases with time as observed in an artificial mouth system (b).

Figure 2. Flow cell system for the visualization and study of biofilm on substratum: custom built at the Oral Health Co-operative Research Centre, Melbourne Dental School, with three inlets and outlets to ensure laminar flow. It has a removable insert for placement of substratum, which can be removed after completion of the experiment.

Figure 3. Constant depth film fermenter modified for the culture of polymicrobial biofilms on enamel substratum (modified from Dashper et al., 2007 [77]). PTFE sampling pans are each modified to hold three enamel substrata recessed to specified depths. The supporting steel disc rotates at 3 rpm and the entire apparatus is placed in a 37^0C incubator. Compositional analysis of the biofilms cultured from the enamel substrata is performed after completion of experiment.

Figure 4. Representative confocal laser scanning microscopy image of a *S. mutans* biofilm cultured in a Stovall flow cell under constant flow of 25 % Artificial Saliva Media for 16 h. The biofilm was then stained with BacLight LIVE/DEAD stain and imaged under a Zeiss

LSM 510 META confocal microscope with a 63 x objective. Image courtesy of Deanne Catmull.

Figure 5. Representative transverse microradiographic images of enamel subsurface demineralised lesions that developed in enamel substrata when exposed to a polymicrobial biofilm cultured in a constant depth film fermenter for 19 days with artificial saliva medium and subjected to regular sucrose pulses. The enamel blocks were painted with acid resistant nail varnish to leave a window (1 X 7 mm) of exposed enamel. The images show subsurface lesion depths at day 6 (15 μm), day 12 (46 μm) and day 19 (82 μm) with adjacent sound enamel protected by the varnish. A 50 μm scale bar is shown at the bottom right of each image.

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