Journal of Dentistry 50 (2016) 1–11

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.intl.elsevierhealth.com/journals/jden

Review article

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

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 4 April 2016
Accepted 24 April 2016

Keywords:
Enamel demineralisation
Oral microbiota
Plaque
Constant depth film fermenter
Microtitre plates
Flow cells

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.

1. 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

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http://dx.doi.org/10.1016/j.jdent.2016.04.010
0300-5712/

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 1000 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 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. 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