Polymicrobial oral biofilm models: simplifying the complex

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Abstract

Over the past century, numerous studies have used oral biofilm models to investigate growth kinetics, biofilm formation, structure and composition, antimicrobial susceptibility and host-pathogen interactions. *In vivo* animal models provide useful models of some oral diseases; however, these are expensive and carry vast ethical implications. Oral biofilms grown or maintained *in vitro* offer a useful platform for certain studies and have the advantages of low cost of establishing such models, as well being easy to reproduce and manipulate. In addition, a wide range of variables can be monitored and adjusted to mimic the dynamic environmental changes at different sites in the oral cavity, such as pH, temperature, salivary and gingival crevicular fluid flow rates, or microbial composition. This review provides a detailed insight for early-career oral science researchers into how biofilm models used in oral research have progressed and improved over the years, their advantages and disadvantages, and how such systems have contributed to our current understanding of oral disease pathogenesis and aetiology.

Introduction

Polymicrobial oral biofilms consist of many bacterial and fungal species. Approximately 700 bacterial species or phylotypes (1) and more than 100 fungal species (2) have been identified in the oral cavity. It is estimated that overall species numbers may well exceed 1000, although many of these are uncultivated (3). There is significant diversity in the oral microbiome, varying greatly from person to person. For example, only 100-200 microbial species are thought to be found in the oral cavity of any given individual (4). Despite this diversity, the concept of microbial “complexes” of microorganisms has emerged, which demonstrates a shift in biofilm colonisation from health to disease, such as in the development of periodontal diseases (5, 6). Our understanding of how dental plaque composition relates to oral health and disease have also changed over time. For example, hypotheses such as the “non-specific plaque hypothesis” (7) “specific plaque hypothesis” (8), “ecological plaque hypothesis” (9) and “keystone pathogen hypothesis” (10) were all developed over the past 50 years. Throughout time, these hypotheses have set the foundations of future oral microbiological research, ultimately contributing to our current understanding of the complex nature behind microbial disease onset and progression in the oral cavity. Recently, OMICs approaches (e.g., genomics, transcriptomics, proteomics and metabolomics) have enhanced our understanding of microbial interactions in the oral cavity, and it is now possible to identify all microbial species that colonise our mouths (3, 11). The OMICs platforms provide the power to investigate complex systems in unprecedented detail, and these have been used to examine biofilms in human diseases and in animal models of disease. Nonetheless, *in vitro* biofilm models provide useful systems for oral microbiologists and immunologists. In the following sections we will discuss how oral biofilm models have advanced over the past century and

appraise their value in understanding the pathophysiology of oral disease throughout each era.

The initial discoveries in oral microbiology – "*the pioneers"*

Over three centuries ago, Antoni van Leeuwenhoek was the first to observe that bacteria resided in the oral cavity. His descriptions of the 'white matter between teeth' (now known as 'dental plaque') and hand-drawn pictures of plaque observed through a homemade light microscope were later found to likely represent *Streptococcus* chains, rod-shaped *Actinomyces* and *Fusobacteria*, and spiralling forms of spirochetes. However, it would not be until the late 19th and early 20th century that scientific research would begin to truly increase our knowledge of the microorganisms that colonise the oral cavity. In 1952, Pigman et al (12) proposed that Magitot was the first to conceive the early ideas for *in vitro* oral microbiology work as far back as 1878, which involved the simple process of incubating extracted teeth in culture media. At a similar time, Black and Miller postulated that accumulation of bacteria in dental plaque facilitated pathogenicity (13, 14), which has now been superseded by modern-day hypotheses e.g., the ecological plaque and keystone pathogen hypothesis. This early work collectively resulted in the identification of cariogenic *Streptococcus* species, including *Streptococcus mutans*, which was named by Clarke in 1924 and later found to play an aetiological role in experimental caries in 1960 by Fitzgerald and Keyes (15). These studies led to an increase in the number of researchers investigating oral bacterial interactions *in vitro.*

A foundation for *in vitro* plaque research - "*the early colonisers"*

Following further confirmation of the causal link between cariogenic streptococci and dental caries during the 1960s (16-18), oral microbiologists took a reductionist approach and turned to assessing the nutritional requirements, culture conditions and plaque forming-capabilities of *S. mutans* and other cariogenic bacteria (19-24). However, most of this work was limited to the use of bacterial culture in planktonic suspension, which we now know does not reflect the true conditions observed in the oral cavity. Such investigations on *S. mutans* led to the development of the "specific plaque hypothesis" by Loesche (8), who proposed that *S. mutans* along with other *Streptococcus* and *Lactobacillus* species were specific pathogens responsible for dental caries. As with most microbial-induced diseases, the search was then on for identifying a 'gold-standard' active compound that could target specific pathogens in the oral cavity. In 1970, Gjermo et al (25) was one of the first to assess the antimicrobial effects of multiple compounds on plaque samples formed *in vivo* and *in vitro.* One of the compounds tested, chlorhexidine gluconate (CHX), is now the main component of many commercial mouthwashes*.* Other studies subsequently showed that CHX was a broad-spectrum anti-septic against most oral microorganisms (26, 27). Unfortunately, due to limitations in microbiological techniques, much of the work at the time was restricted to utilising undefined plaque samples with unknown composition, meaning reproducibility was low, or largely unachievable. An improvement in culture-based methods in the 1970s, including the development of anaerobic jars and cabinets, expanded the breadth of *in vitro* research. The ability to grow strict anaerobes led to the identification of pathogens associated with PD. Consequently, a range of anaerobic bacteria such as *Bacteroides gingivalis* (now known as *Porphyromonas gingivalis*) were isolated from PD lesions (28). Research throughout

this period concentrated on producing robust methods for isolating and cultivating anaerobic bacteria from plaque (29, 30). As a result, the “non-specific plaque hypothesis” and “specific plaque hypothesis” were adjusted to take into account specific anaerobic pathogens associated with PD (7, 31).

An advancement in microscopic techniques also meant that *in vitro* oral research began to flourish throughout the 1970s. The first scanning electron microscope (SEM) and transmission electron microscope (TEM) were made commercially available during this time, meaning that the morphology and architecture of individual colonies and cell aggregates in plaque could be imaged. Publications by Sudo and colleagues (32, 33) described the development of a model to assess continuous culture of saliva samples on substrates of glass beads coated with hydroxyapatite (HA) to mimic the tooth surface. SEM images provided evidence of bacterial colonization on the HA-coated beads with distinct cell-cell interactions between different bacterial species (33). Others followed suit with multiple studies reporting the use of SEM and TEM technology to image plaque formed both *in vivo* and *in vitro* (34-36). It was arguably the combination of culture-based methods and microscopy at the time that helped us to understand the complex polymicrobial interactions between different species in plaque. Accordingly, it was at a similar time that the phrase “biofilm” was initially coined by Costerton in 1978 (37), a term that is now synonymous with oral microbiology.

Bridging the gap with chemostat models – "*the intermediate colonisers"*

Multiple research groups reacted to this new way of considering oral microbial plaque and started to utilise continuous cultures to generate "biofilms" under appropriate controlled conditions (e.g., under shear force and constant flow). This technique, which utilises a chemostat flow system, allows for the growth of planktonic cultures and biofilms through a regular supply of fresh medium, continuously pumped through the system, with spent growth medium being removed at a similar rate (Figure 1A). The idea of the chemostat was first described in 1950 as a method to culture a bacterial population at a reduced growth rate (38, 39). Continuous culture models were revolutionary for oral biofilm research although most early work using the technology was restricted to planktonic mono- or mixed-cultures. Throughout the 1970s, the metabolic and enzymatic activity of cariogenic microorganisms were assessed following growth in chemostat systems pumped with different carbohydrates such as sucrose, fructose and glucose (33, 40-42). However, it was undoubtedly the pioneering work of Marsh who instigated the use of the chemostat in oral microbiology, driving the field forward during the 1980s. His early chemostat work was limited and was used to measure the growth rate of 1-2 bacterial species or undefined plaque samples in nutrient-excess or limited media (43, 44). However, later in the decade, defined mixed-species containing a more complex consortium of anaerobic microorganisms such as *Fusobacteria*, *Actinomyces*, *Veillonella*, *Neisseria* and *Bacteroides* species were introduced into chemostat systems under standardised conditions. Whilst reductionist in nature, these studies were some of the first to piece together interactions between different species which relatively reflected *in vivo* plaque, and subsequently led to the formulation of the "ecological plaque hypothesis"

(9). Of note at the time, the works of McKee et al (45), McDermid et al (46) and Bradshaw et al (47) all demonstrated that mixed-species cultures were influenced by carbohydrate availability, and subsequent shifts in pH. In these studies, cultures were pulsed with carbohydrate-rich media resulting in the generation of an acidic environment (following carbohydrate fermentation by cariogenic microorganisms), before the pH was adjusted back to neutral. This technique ultimately replicates the environment of the oral cavity after a consumption of a carbohydrate-rich meal, but also accounts for the return to a relatively neutral pH between meals. Additionally, as the pH at certain oral sites may be slightly alkaline (48, 49), chemostats provided an excellent platform for growing mixed-species biofilms in a highly controlled manner, in environments that closely mimic different sites of the oral cavity.

By the late 1980s and early 1990s, chemostat systems were utilised for biofilm growth, as well as assessing the impact of nutrient availability on plaque composition and in antimicrobial susceptibility biofilm testing (50-53). These flow-through models allowed for minimum inhibitory concentration (MIC) testing of anti-microbials such as triclosan, CHX, xylitol and fluoride against mixed-species biofilms. An advantage being that the compounds could be pulsed through the system using MIC or sub-MIC levels, or dosed appropriately to give a constant final concentration. This meant that the effects of such compounds could be assessed either directly on bacterial species, or indirectly, if inhibition of one species impacting on the growth of another, thus altering the homeostasis of the biofilm. In 1991, Marsh and colleagues (54) showed that *S. mutans*, *Lactobacillus casei* and *Veillonella dispar* (carbohydrate-fermenting, acid-tolerant bacterial species) predominate a mixed-species biofilm in environments of carbohydrate excess, and subsequent acidic pH, whilst treatment with sodium fluoride

reduced the acid production, stabilising the biofilm and allowing other bacterial species to flourish. Similar mechanisms were proposed for pathogens associated with PD, with Bradshaw and colleagues demonstrating that anaerobic bacteria such as *Fusobacterium nucleatum* and *P. gingivalis* could only survive aerobic environments when grown in communities containing oxygen-consuming bacteria (55-57). It was around this time that Marsh proposed the “ecological plaque hypothesis”, which assumed that an imbalance in the composition of the oral biofilm due to environmental factors (carbohydrate accessibility and pH in dental caries), and nutrient availability (oxygen levels and redox potential in PD) can result in the enrichment of disease-associated pathogens (9). Although chemostats do have evident disadvantages e.g., high risk of contamination and low throughput, the use of such systems provided an opportunity to mimic *in vivo* environments with higher accuracy than previously developed models.

Throughout the 1990s, the concept of chemostat flow was utilised and adapted for other continuous culture systems. A constant depth film fermenter (CDFF) used the principle of chemostat flow to reproducibly grow a large mass of biofilm with defined thickness for imaging, antimicrobial testing and inter-species transfer of drug resistance genes (58-61). Flow cells, which are slides containing suitable substrata such as HA, were also introduced into chemostat systems to assess biofilm formation and allow for microscopic imaging of the biofilm development. In 1994, Herles and colleagues (62) utilised a chemostat-flow cell system to study the effects of different anti-plaque agents on biofilms formed on flow cells. Their work qualitatively showed that multi-species biofilms formed *in vitro* were susceptible to triclosan-containing mouth-rinse, whilst untreated biofilms remained largely viable. Interestingly, at a

similar time, Singleton et al (63) combined flow cell technology with fluorescence microscopy to map spatial distribution of bacterial species within plaque using mathematical modelling. Others have used flow-cell systems in conjunction with fluorescence microscopy for imaging interactions between organisms (64-66). Cook, Costerton & Lamont (64) were the first to show that *Streptococcus gordonii* forms an attachment substratum on saliva-coated flow-cell coverslips for *P. gingivalis* using confocal laser scanning electron microscopy (CLSM). Thus, fluorescence microscopy highlighted key microbial interactions like never before.

Maturation of modern-day models – "*the late colonisers*"

Microbiological models – from plaque to plate

Static biofilm models became important systems in the field of oral microbiology during the 1990s. These models exploited a relatively simple method of producing biofilms to perform high-throughput antimicrobial susceptibility testing and phenotypic screening of mutant libraries to assess the importance of certain genes in biofilm formation (67-69). Oral biofilms were grown on either plastic, glass, HA coated-substrates, directly in the bottom of different sized microtiter plates, or on inverted pegs placed into specific media with inoculum (Figure 1B). The latter method known as the Calgary Biofilm Device (CBD) or minimum biofilm eradication concentration (MBEC) assay was first described by Ceri et al in 1999 (70). The CBD method remains used to this day, particularly for antimicrobial testing on undefined plaque samples cultured *in vitro* on inverted pegs (71-73). One major advantage of these static models is that multi-species biofilms can be grown in large quantities, an improvement of chemostat flow systems which were restricted to producing no more than a few biofilms at any given time. In the present day, modern high-throughput techniques for oral biofilm cultivation

have been developed, e.g., microfluidics for miniaturising biofilm culture and characterisation (74-76) and impedance-based technology for real time monitoring of biofilm growth (77) (Figure 1C). However, these new techniques are expensive and therefore batch methods for growing biofilms in microtiter plates are arguably the preferred choice for the majority of research groups given the cost attached to generating such models. Additionally, these high-throughput approaches serve as ideal models employed by industry, which seek robust, minimalistic biofilm platforms that can be used as definitive test-beds for standardised biofilm testing (78, 79). Advantages and disadvantages of the continuous flow models, static biofilm systems and current high-throughput modern-day options utilising new technology are discussed in greater depth in Table 1.

From the turn of the millennium, many research groups began to utilise high-throughput static systems to develop their own multi-species biofilms from a defined number of microorganisms (Table 2). The increase in commercially available real-time quantitative polymerase chain reaction (RT-qPCR) machines in the early 2000s made this possible, meaning oral biofilm models could be grown to precise composition. Previously, compositional analysis was largely restricted to using selective and differential media for identification of different microbial species grown in multi-species biofilms. Currently, the use of genus- or species-specific primers in conjunction with microscopic technology means that biofilms can be repeatedly grown with reproducible composition and architecture (Figure 2A) (80). In addition, it is now possible to discriminate between viable or dead microbial species in biofilms using RT-qPCR methods. In 2011, Loozen et al (81) described a protocol using a DNA intercalating substance called propidium monoazide to identify the proportion of dead

S. mutans, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* cells in heat-killed mono-cultures. Since development, this method has become an important tool in assessing the effectiveness of antimicrobials and other actives on oral biofilms (82, 83)

Immunology based models – the host-biofilm interface

In the last two decades, oral immunologists have started to utilise these defined multi-species biofilms to identify mechanisms involved in oral disease pathogenesis and aetiology. As such, co-culture mammalian cell-biofilm models are now frequently used in oral health and disease research (Figure 2B). Co-culture models have been described using primary and immortalised cell lines (e.g., immune cells including T cells, B cells, neutrophils and monocytes, primary human oral or gingival epithelial cells and immortalised cell lines such as OKF4, OKF6/TERT2 or TR146) and multi-layered tissues (e.g., in-house 3D tissue models containing keratinocytes and fibroblasts or commercially available organotypic tissue). Results from multiple research groups have shown that cell viability and immune response in orally relevant cell lines or tissues can vary depending on biofilm complexity, composition and viability (84-86). These studies have demonstrated that commensal and pathogenic microbial species differentially modulate the epithelial immune response. For example, oral epithelial cells challenged with biofilms containing only commensal, health-associated microorganisms result in a minimal pro-inflammatory response, whilst biofilms comprised of disease-associated pathogens can induce an elevated pro-inflammatory gene- and protein- signature and induce cytotoxicity. It was *in vitro* co-culture research that showed planktonic *P. gingivalis* can invade oral epithelial cells and impair cytokine production (87-89). For example, interleukin-8 (IL-8) production is diminished by a

serine phosphatase and degraded by the cysteine proteases called gingipains produced by *P. gingivalis* (90, 91). Others have shown that oral epithelial cells co-cultured with *P. gingivalis*-containing biofilms result in reduced IL-8 protein detection in spent cell supernatants (85, 92). Cytokine degradation may explain the "local chemokine paralysis" by which *P. gingivalis* delays the recruitment of neutrophils to the gingival tissue, impairing the mucosal defence to the pathogen *in vivo*, and ultimately evading the immune system (87). The plethora of oral biofilm and cell co-culture models now available means that it is possible to investigate the mechanistic pathways that oral pathogens (and commensals) utilise to facilitate inflammation in a highly controlled manner *in vitro*. Despite their simplicity, these *in vitro* studies provide a strong foundation for on-going *in vivo* research.

Imaging biofilms – Spying on the community

Microscopy techniques have advanced in recent years; light, fluorescent and electron microscopic technology are now common methodologies for imaging oral biofilms in most laboratories (Figure 2C). In addition, these methods, in particularly fluorescent microscopy, have been adapted for more unique investigations in oral biofilm research. For example, an interesting technique described by Schlafer et al (93) utilising a ratiometric pH-sensitive dye called C-SNARF-4 has been applied to oral biofilms to monitor changes in pH in the biofilm landscape. A five-species early dental plaque biofilm containing four *Streptococcus* species and *A. naeslundii* was grown in the presence and absence of artificial saliva containing 0.4% glucose, and pH gradients in the extracellular matrix monitored fluorescently. Regions within the biofilm with higher cell density correlated with lower pH values (93).

Fluorescent *in situ* hybridization (FISH), a technique allowing for sensitive identification of target microorganisms within complex microbial communities, has been used in imaging of oral biofilms, sampled directly from volunteers, or grown *in vitro*. Thurnheer, Gmur and Guggenheim (2004) were the first to combine FISH and CLSM to stain six species of bacteria and fungi within *in vitro*-grown oral biofilms (94). All six microbial species were identified using fluorescently labelled 16S or 18S rRNA-targeted oligonucleotides, with biofilms containing *S. oralis*, *Streptococcus sobrinus*, *Veillonella dispar*, *F. nucleatum*, *Actinomyces naeslundii* and *Candida albicans* visualised from two experiments (e.g., two independent triple-hybridisations using three different fluorescent probes). Other publications have used similar methods to show localisation of different bacterial species in mixed-species biofilms using multiple independent hybridisations (80, 95). A publication by Kommerein et al (2017) effectively showed the development of a robust four-species biofilm model with highly reproducible biomass and architecture as assessed by FISH (80). It is now possible to use additional combinations of fluorophores to identify tens of bacterial species in dental plaque. A publication by Valm (96) elegantly described an “proof of concept” imaging technique which combines fluorescent labelling coupled with spectral image acquisition and analysis to identify 15 different oral taxa in dispersed dental plaque. The technology, which is known as Combinatorial Labelling and Spectral Imaging (CLASI-FISH), has scope to distinguish between 120 different fluorescently-labelled microorganisms in a single image (97). In 2016, Mark-Welch et al (98) used CLASI-FISH to visualise the “biogeography of the human oral microbiome” in intact dental plaque. The study utilised a nine- and ten-probe set of fluorescently labelled probes to demonstrate that oral biofilms formed unique ‘hedgehog-like’ structures predominated by *Corynebacterium* filaments that provide a “scaffold” for other microorganisms such

as *Streptococcus* and *Porphyromonas* species. To date, no studies have investigated the feasibility of identifying over 100 microorganisms in dental plaque using CLASI-FISH, nor have any studies applied the technology to oral biofilms grown *in vitro*. However, it would be of great importance to assess whether microbial interactions in laboratory-grown polymicrobial biofilms recapitulate such complex spatial organisations in plaque formed *in vivo*.

Spreading into other technologies – "*biofilm dissemination"*

Throughout the last 20 years, the use of high throughput technologies has become routine to support biological discoveries and drive hypotheses. OMICs approaches have enabled researchers to view the genomics, transcripts, metabolites and proteins of oral biofilm models in a "holistic" manner.

Microbiome analysis by shotgun sequencing or 16S amplicon sequencing has become one of the most widely used technologies and bioinformatic techniques in the microbiologist's toolbox. Within the last 10 years there has been an increase in microbiome studies due to the cost of the sequencing technologies decreasing and the availability of analysis pipelines and packages increasing (99-101). The microbiome has been studied in caries, PD, denture stomatitis, peri-implantitis among several other oral diseases (102-105). These studies have allowed for the greater in-depth characterisation of distinct microbial community shifts within the oral cavity, also accounting for uncultivated organisms. Large-scale molecular techniques such as microbiome sequencing have allowed for the identification of species, as well as species-species interactions and environment-species interactions previously undeterminable with conventional techniques. In periodontitis, such techniques have

uncovered unique characteristics exhibited by the sub-gingival plaque biofilm in health and disease. For example, Abusleme *et al.,* (106) highlighted that in contrast to infections occurring in other sites of the body where diversity has been shown to decrease during active disease, in periodontitis, the diversity of sub-gingival plaque significantly increases in relation to health. Similarly, analysis of the sub gingival plaque microbiome has revealed that disease associated organisms may extend far beyond the previously identified 'Red-complex'. Of note, organisms such as *Filifactor alocis* and *Prevotella denticola* have been consistently found to be higher in diseased sites (107-109). Together, such analysis has led to the re-evaluation of individual pathogens driving a disease state, and it is now hypothesised that "ecotypes" and/or whole microbial communities may drive dysbiosis and pathogenesis (110, 111).

The sequencing of messenger RNA for studying transcriptomics is a powerful tool to gain insight into how an organism is reacting to and adapted to its environment. This can give us clues and hypotheses about how microorganisms interact in a complex biofilm environment. For example, the use of RNA-Seq has helped in our understanding of species-species adhesion and virulence mechanisms between *C. albicans* and *P. gingivalis* (112), and synergistic relationships in *C. albicans* and *S. mutans* biofilms (113). Transcriptomic studies have also been indispensable in understanding disease pathogenesis, drug resistance mechanisms and community dynamics with regard to oral biofilm research in recent years (114, 115). In a study by Frias-Lopez et al (114), it was shown that incorporation of periodontal pathogens (e.g., *P. gingivalis*) into biofilms containing commensal species can alter patterns in gene expression in the healthy oral community.

Similarly, Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) have allowed for snapshot profiling of metabolic and protein expression for the identification of signatures in oral biofilms. Metabolomics enables the identification and relative quantification of all the metabolites in a biological system. Metabolomic pathways and biofilm regulatory mechanisms are discernible with these technologies (116). They offer the potential to identify signatures associated with disease progression. This was highlighted in a 2015 pilot study which validated the use of metabolomics by Gas Chromatography–MS in identifying metabolic profiles between cariogenic and disease-free oral biofilms (117). Advancements have also been made in oral microbial studies which allow for the correlation of the metabolome with shifts in the oral microbiome (110). These studies further our understanding of bacterial communities with different ecological states with more specialised function, which the authors of the 2017 study (110) suggest could highlight specific metabolic function of communities which could indicate dysbiosis. In the coming years, it is likely that the use of OMICs approaches in investigating microbial-host interactions will continue to grow. It is important that these techniques should be utilised in conjunction with laboratory science, in order to identify mechanistic pathways by which oral pathogens facilitate disease onset and progression.

Conclusion

We have highlighted in this review how our understanding of oral microbiology at any given time has resulted in relevant robust biofilm models, often simple to start with, but developing complexity over time. On the contrary, *in vitro* biofilm models have also enhanced our knowledge of the simple and complex microbial interactions in the oral cavity. Recent OMICs analyses imply that no oral biofilm model is ideal, and that it is

simply impossible to replicate exact microbial-microbial or microbial-host interactions *in vitro*. Nonetheless, understanding how a few organisms interact together in biofilms, and with host immunity, aids our view of microbial and host dynamic interactions. We should not lose sight of the importance of hypothesis-driven research when creating or using these models, and not simply rely on 'big data' to shape our ideas and become more speculative in our outlook. As with all approaches, OMICs techniques are not devoid of disadvantages. Whilst offering powerful platforms to investigate a large number of variables simultaneously, issues persist over poor reproducibility and statistical bias. As such, there remains a requirement for such approaches to be supplemented with accessible and reproducible *in vitro* assays. In the future, combining results from OMICs studies, *in vivo* animal models, and *in vitro* biofilm research will undoubtedly continue to enhance our knowledge of oral disease aetiology and pathogenesis, consequently impacting clinical practice and disease treatment.

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Figures and Tables

Figure 1 – Models used to grow oral biofilms. Early work focused on the use of continuous flow systems such as the chemostat model which offered the advantage of a regular supply of fresh medium whilst maintaining a constant media volume (A). However, such models were hindered by low-throughput, leading to the development of high-throughput static biofilm models (B). Since development, static biofilms have become the most ubiquitously used models in oral research and encompass the conventional model; where biofilms are grown in microtiter plates, and the Calgary device; where biofilms are grown on pegs attached to the surface of lids. As the field of biofilm research continues to grow, novel systems have been employed to incorporate the advantages of continuous flow and static models. One such model is the microfluidic device, which uses microchannels to combine continuous media flow with high-throughput screening potential (C). Image created by Biorender.

Figure 2 - Different methodologies used to investigate oral biofilms. There are three main outputs used to generate data from biofilm models described in this review; microbiological, immunological and microscopic methods. Modern investigations seek to analyse the composition of *in vitro*-grown polymicrobial biofilms using quantitative PCR and species- or genus- specific primer sets, whilst omics approaches have been used to investigate the unique transcriptome profiles of microorganisms associated with health and disease (A). Co-culture systems are now commonplace in oral biofilm research. These involve the simultaneous incubation of biofilms with immune cell types such as gingival epithelial cells to assess host-pathogen interactions and better understand the underlying pathologies of oral disease (B). Microscopic techniques include the use of simple light microscopy to observe general organism topography within biofilms, fluorescent microscopy (e.g. FISH) to highlight organism viability and scanning electron microscopy (SEM) to visualise distinct cell-cell aggregates encapsulated within a biofilm (C). Image created by Biorender.

Model	Description	Advantages	Disadvantages
Continuous flow models			
Chemostat	A bioreactor system with a continuous inflow of fresh media and sterile air, waste media is removed at the same rate to maintain a constant culture volume. Biofilms form within the culture chamber.	Suitable for growth of large biofilms. Continuous flow of fresh media. Allows for control of the external environment which can influence biofilm growth.	Low through-put. Requires specialised equipment. Large quantity of material lost if contaminated. No direct access to biofilms. Prone to contamination.
Flow-cell	Slides containing suitable substrata which allow biofilms to form within small channels under a constant flow of media. Spent media is removed	Allows for direct visualisation of biofilm growth stages. Optimised for microscopy. Compatible with a range of substrata. Continuous flow of fresh media.	Low through-put. Requires specialised equipment. No direct access to biofilms. Prone to contamination.
Constant depth film fermenter	Coupons are suspended from the lid via a suitable coupon holder. Fresh media is pumped through the reactor, with continuous mixing. Biofilms form on the surface of coupons.	Commercially available. Continuous flow of fresh media. Allows for direct access to biofilms. Can alter shear stress.	Low through-put Requires specialised equipment. Expensive. Prone to contamination.
Static models			

Microtiter plate	Media containing bacteria is placed into microtiter plate. Biofilms form on the bottom of wells, or on suitable substrates placed in each well. Media can be replaced through pipetting.	High through-put. Inexpensive. No specialised equipment required. Microtiter plates with varying well sizes can be used to better suit the desired assay.	Biofilms not grown under continuous flow. Prone to sedimentation. Multiple plates required for continuous timepoint assays. Labour intensive.
Calgary device	Media containing bacteria is placed into 96-well microtiter plates. Pegs suspended from lids are placed into wells, allowing for biofilm formation on their surface.	High through-put Allows for easy manipulation of culture media. Requires active attachment of bacteria as opposed to sedimentation.	More expensive than simple microtiter plates. Multiple plates required for continuous timepoint assays. Prone to contamination. Labour intensive.
Modern technologies			
Microfluidics (e.g., BioFlux microfluidic devices)	Small microfluidic chips contain channels etched into a suitable substrate (glass, silicon, plastic) which are connected through inlets and outlets. Media is pumped through channels, allowing for micro-biofilm formation.	High through-put. Can be designed to mimic specific environments. Suitable for antimicrobial susceptibility testing. Allows for analysis of different stages of biofilm growth.	Expensive. Requires specialised equipment for preparation and analysis. Clogging may occur in micro-channels.

Impedance-based technology (e.g., XCELLigence real-time monitoring system)	Media containing bacteria is placed into microtitre E-plates with gold electrodes on the plate floor. Electron impedence is used to monitor several factors such as biofilm growth and cell morphology. Cell index values directly related to biofilm maturation.	High through-put. Allows for real-time monitoring of biofilm growth. Suitable for antimicrobial susceptibility testing. Can allow for direct access to the biofilm.	Expensive. Requires specialised equipment for preparation and analysis. Prone to sedimentation. Gold electrodes can be fragile and easily damaged.

Table 1 – Advantages and disadvantages of current oral biofilm methodologies. A table showing the current methodologies used by research groups for growth of oral biofilm models including continuous flow systems, static models and current modern options utilising new technology. A description of the models, advantages and disadvantages are highlighted for each methodology.

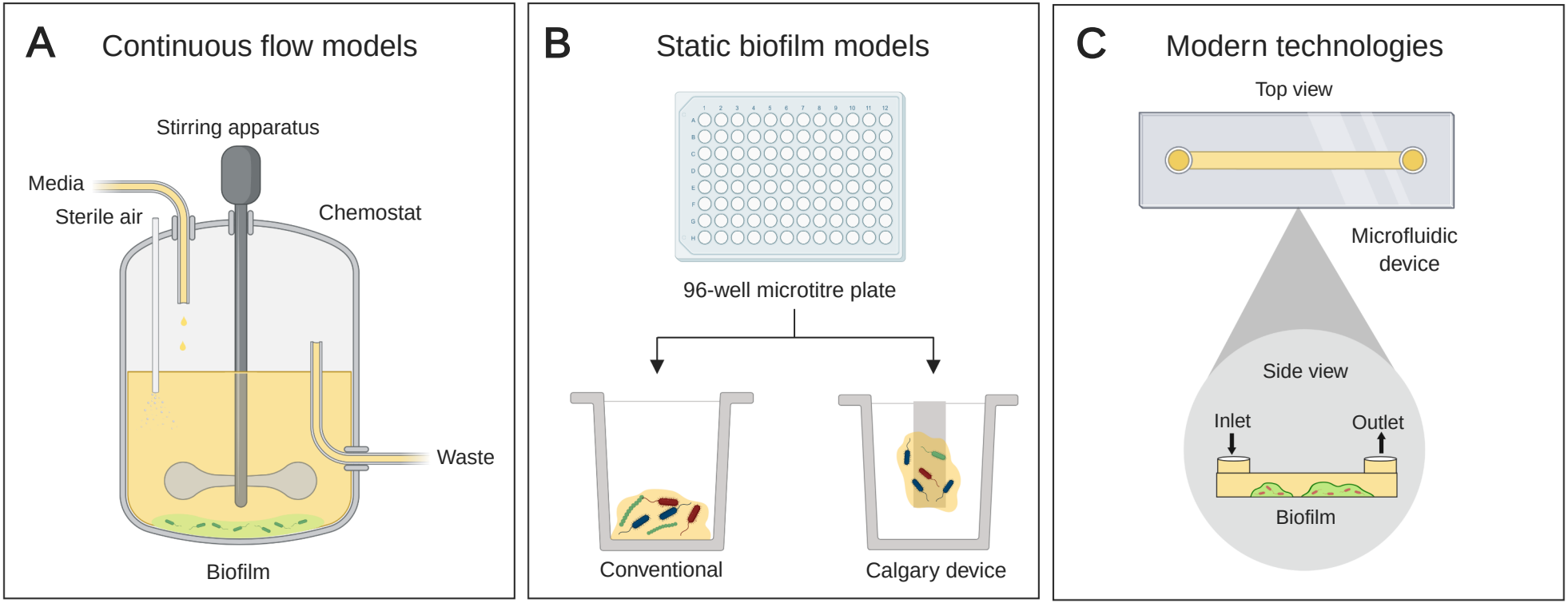
Authors	Biofilm	Bacterial/fungal strains	Substratum	Media	Reference
Guggenheim et al., 2001 *	Supra-gingival biofilm	*Streptococcus oralis, Streptococcus sobrinus, Veillonella dispar, Actinomyces naeslundii & Fusobacterium nucleatum*	Saliva-coated hydroxyapatite disc	Human saliva, human serum and modified universal fluid medium	(118)
Guggenheim et al., 2009 * and Ammann et al., 2012	Sub/Supra-gingival biofilm	*Streptococcus intermedius, Streptococcus oralis, Campylobacter rectus, Fusobacterium nucleatum spp. vincentii, Veillonella dispar, Actinomyces naeslundii, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia & Treponema lecithinolyticum* or *Treponema denticola*	Saliva-coated hydroxyapatite disc	Human saliva, human serum and modified universal fluid medium	(95, 119)
Peyyala et al 2013 *	Health, gingivitis, periodontitis biofilms	*Streptococcus gordonii, Streptococcus oralis & Streptococcus sanguinis* (health), *Streptococcus gordonii, Actinomyces naeslundii & Fusobacterium nucleatum* (gingivitis), *Streptococcus gordonii, Fusobacterium nucleatum* & *Porphyromonas gingivalis* (periodontitis)	Rigid gas permeable hard contact lenses	Supplemented brain heart infusion broth	(84)
Frias-Lopex and Pinedo, 2012	Health, periodontitis biofilms	*Actinomyces naeslundii, Lactobacillus casei, Streptococcus mitis, Veillonella parvula & Fusobacterium nucleatum* (health)	Saliva-coated hydroxyapatite disc	Mucin growth medium	(114)

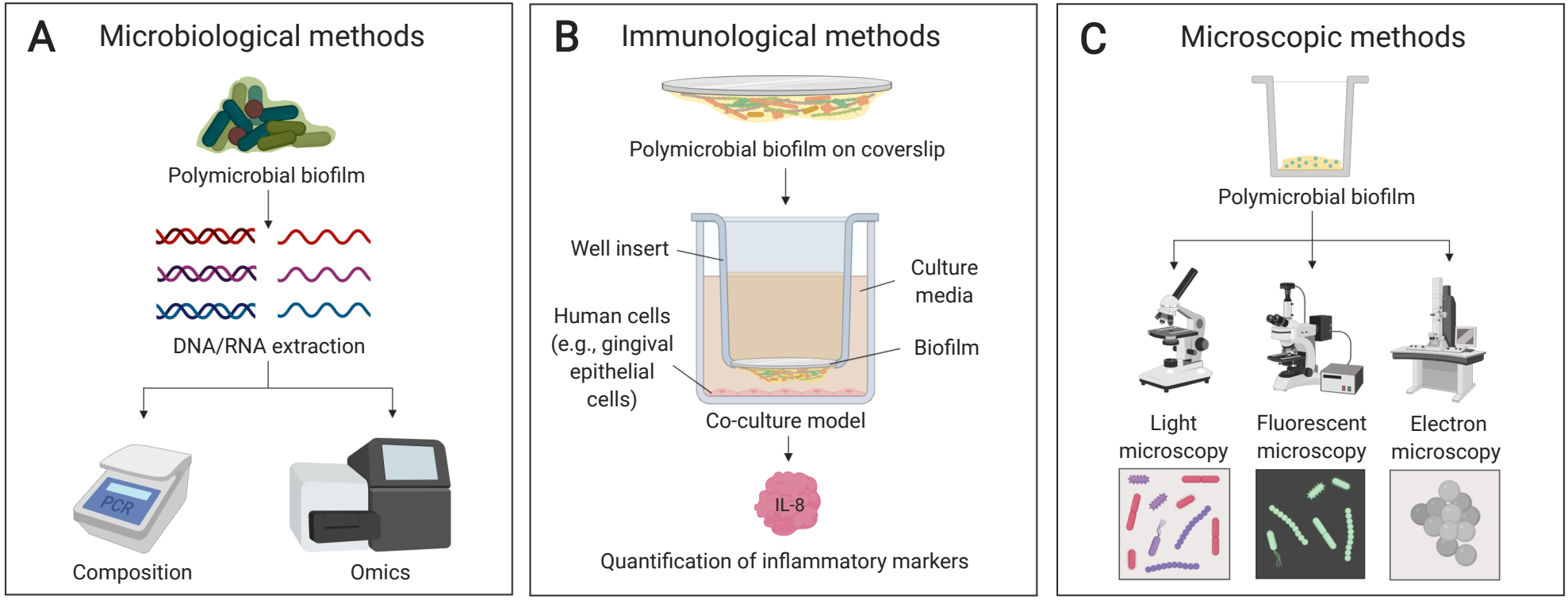
		including *Porphyromonas gingivalis & Aggregatibacter actinomycetemcomitans* (periodontitis)			
Falsetta et al 2014	Caries biofilm	*Streptococcus mutans & Candida albicans*	Saliva-coated hydroxyapatite disc	Human Saliva	(120)
Millhouse et al 2014	Periodontitis biofilm	*Streptococcus mitis, Fusobacterium nucleatum, Porphyromonas gingivalis & Aggregatibacter actinomycetemcomitans*	Thermanox™ coverslips	Artificial Saliva	(121)
Muhammad et al 2014	Endodontic biofilm	*Enterococcus faecalis, Streptococcus salivarius, Porphyromonas gingivalis & Prevotella intermedia*	Extracted tooth	Schaedler anaerobic broth	(122)
Cavalcanti et al 2016	Peri-implantitis biofilm	*Candida albicans, Streptococcus mutans, Streptococcus oralis, Veillonella dispar, Actinomyces naeslundii & Fusobacterium nucleatum*	Titanium coated discs	Modified universal fluid medium with glucose	(123)
Sherry et al 2016	Denture stomatitis biofilm	*Candida albicans, Streptococcus intermedius, Streptococcus mitis, Streptococcus oralis, Aggregatibacter actinomycetemcomitans, Actinomyces naeslundii, Veillonella dispar, Fusobacterium nucleatum, Fusobacterium nucleatum spp. vincentii, Porphyromonas gingivalis & Prevotella intermedia*	Polymethylmethacrylate discs	Artificial Saliva	(83)

Thurnheer & Belibasakis, 2016	Peri-implantitis biofilm	*Streptococcus oralis, Streptococcus anginosus, Actinomyces oris, Fusobacterium nucleatum, Veillonella dispar, Campylobacter rectus, Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Staphylococcus aureus & Staphylococcus epidermidis*	Saliva-coated hydroxyapatite or titanium discs	Human saliva, human serum and modified universal fluid medium	(124)
Kommerein et al., 2017	Peri-implantitis biofilm	*Streptococcus oralis, Actinomyces naeslundii, Veillonella dispar Porphyromonas gingivalis*	96-well glass bottom plate	Supplemented brain heart infusion broth	(80)
Zhou et al., 2018	Caries biofilm	*Streptococcus mutans, Lactobacillus casei*, *Veillonella dispar, Actinomyces naeslundii & Fusobacterium nucleatum*	Thermanox^{TM} coverslips	Artificial Saliva	(125)
Ramage et al., 2019 *	Denture Stomatitis	*Streptococcus mitis, Streptococcus intermedius, Streptococcus oralis, Candida albicans, Actinomyces naeslundii, Veillonella dispar, Rothia dentocariosa, Lactobacillus casei & Lactobacillus zeae*	Polymethylmethacrylate discs	Artificial Saliva	(126)

Table 2 – The multi-species oral biofilm static models developed over the last 20 years. A table showing the range of oral biofilm static models developed by multiple research groups. Each model contains bacterial and fungal species associated with different health and disease states in the oral cavity. Asterisks denote models of major importance (*). In 2001, Guggenheim et al (118) was one of the first to describe the validation of a supra-gingival static biofilm model. Prior models to this work predominantly

utilised continuous culture systems such as chemostats or flow cells. The “Zurich” biofilm model was adapted by the same group (95) and made more complex including a total of 9- species associated with health and periodontitis. Using a combination of microbiological and microscopic techniques, the authors were able to comprehensively characterise the composition and architectural features of the biofilm, in addition to assessing the inflammatory outputs in a co-culture system with gingival cells. A number of studies have used the “Zurich” biofilm as a template for their own models. The Ebersole group (84) developed biofilm models for co-culture experiments, to study the host-pathogen response to biofilms containing a consortium of microorganisms associated with oral health and disease. The ensuing studies from the group have enhanced our understanding of the host response to commensal and pathogenic biofilms. A recent publication by Ramage et al (126) developed a complex denture plaque model using the dominant microbial genera from a microbiome study (104). Future models may serve to utilise OMICs approaches such as microbiome analyses to develop new biofilm models incorporating microorganisms newly associated with oral diseases such as denture stomatitis, periodontitis or endodontic infections.


A
Continuous flow models
Stirring apparatus
Media
Sterile air
Chemostat
Waste
Biofilm
B
Static biofilm models
96-well microtitre plate
Conventional
Calgary device
C
Modern technologies
Top view
Microfluidic device
Side view
Inlet
Outlet
Biofilm


A
Microbiological methods
Polymicrobial biofilm
DNA/RNA extraction
PCR
Composition
Omics
B
Immunological methods
Polymicrobial biofilm on coverslip
Well insert
Culture media
Human cells (e.g., gingival epithelial cells)
Biofilm
Co-culture model
IL-8
Quantification of inflammatory markers
C
Microscopic methods
Polymicrobial biofilm
Light microscopy
Fluorescent microscopy
Electron microscopy