DEVELOPMENT OF AN INTRAORAL DEVICE FOR *IN SITU* BIOFILM STUDY VALIDATED BY ITS EFFECTIVE APPLICATION IN ASSESSING BIOFILM CHARACTERISTICS

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DEVELOPMENT OF AN INTRAORAL DEVICE FOR *IN SITU* BIOFILM STUDY VALIDATED BY ITS EFFECTIVE APPLICATION IN ASSESSING BIOFILM CHARACTERISTICS

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

ACE	Abundance-based coverage estimator
ANOVA	Analysis of variance
bp	Base pair
BHI	Brain heart infusion
CO_2	Carbon dioxide
CHX	Chlorhexidine
CFU	Colony forming unit
CLSM	Confocal laser scanning microscope
ºC	Degree Celsius
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EO	Essential oil
EMA	Ethidium monoazide
FISH	Fluorescence *in situ* hybridisation
GIC	Glass ionomer cement
g	Gram
hr	Hour
IDOD	Intraoral device of overlaid disk holding splint
<	Less than
≤	Less than or equal to
µL	Microliter
µM	Micromolar
mg	Milligram
ml	Milliliter
mm	Millimeter
mmol/L	Millimole per liter
min	Minute
>	More than
≥	More than or equal to
NGS	Next generation sequencing
OTU	Operational taxonomic units
%	Percentage
PCR	Polymerase chain reaction
KI	Potassium Iodide
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PMA	Propodium monoazide
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RAD	Rank abundance distribution
RNA	Ribonucleic acid

SEM	Scanning electron microscope
SDF	Silver diamine fluoride
Ct	Threshold cycle
TEM	Transmission electron microscope
UPGMA	Unweighted pair group method with arithmetic mean
ZnCl	Zinc chloride

PEMBANGUNAN ALAT INTRAORAL UNTUK KAJIAN BIOFILM *IN SITU* DISAHKAN OLEH KEBERKESANAN PENGGUNAANNYA DALAM MENILAI CIRI-CIRI BIOFILM

ABSTRAK

Pemodelan dan pencirian pengembangan biofilm oral *in situ* telah memainkan peranan penting, terutama dalam kariologi, dalam memahami pathogenesis dan pencegahan penyakit serta pembangunan produk unggul untuk pengendalian karies. Walau bagaimanapun, model yang sedia ada bagi tujuan ini jauh dari memuaskan. Oleh itu, objektif kajian ini yang telah dilakukan dalam dua fasa, iaitu fasa pertama untuk membangun dan memvalidasikan model intraoral untuk pembentukan biofilm *in situ* pada subjek manusia, dan kedua, untuk menilai kesan dua tekanan kimia berbeza iaitu "silver diamine fluoride" (SDF) dan kalium iodida (KI) yang digunakan secara klinikal untuk kawalan karies, pada biofilm plak *in situ* yang dibentuk, menggunakan model yang divalidasikan. Dalam fasa pertama, sebuah model dari bahan akrilik membawa beberapa mangkuk yang boleh ditanggalkan dan boleh dipakai dengan selesa oleh subjek telah dihasilkan. Untuk memvalidasikan model, kajian *in vivo* dilakukan untuk membandingkan viabiliti mikrobial dan komposisi antara biofilm yang terbentuk di dalam model dan gigi menggunakan tindakbalas rantaian polimerase viabiliti atau "viability polymerase chain reaction" (vPCR) dan penjujukan generasi seterusnya atau "next generation sequencing" (NGS). Ciri-ciri struktur biofilm yang dibentuk melalui model dianalisis dengan mikroskopi pemindaian laser confocal atau "confocal laser scanning microscopy" (CLSM). Pada fasa kedua, model tersebut, yang telah divalidasi, digunakan untuk membandingkan keberkesanan antibakteria SDF dan KI sama ada secara tunggal atau dalam kombinasi, pada biofilm plak yang sudah dibentuk dan diseragamkan oleh model baharu. Beberapa sampel biofilm plak *in situ*

yang dibentuk dengan model dikumpulkan dari enam subjek yang sihat selama 4 hingga 24 jam, untuk menyiasat pelbagai hasil. Model unggul ini menjimatkan, mudah dibuat, dan mudah dimasukkan ke dalam mulut dan dikeluarkan oleh subjek. Hasil keputusan menunjukkan tidak ada perbezaan kuantitatif yang signifikan dalam bakteria hidup antara biofilm yang terbentuk dari model dan gigi (p>0.05).Data menunjukkan tidak ada perbezaan yang signifikan antara biofilm plak dan gigi yang terbentuk berkaitan dengan lokasi mangkuk. Walau bagaimanapun, perbandingan antara subjek menunjukkan perbezaan yang signifikan dalam semua parameter viabiliti yang dinilai (p<0.05). Seterusnya, analisis 24 jam biofilm yang dibentuk model *in situ*, di bawah CLSM menunjukkan konfigurasi biofilm heterogen. Pada analisis NGS, sejumlah 10 genera bakteria utama dikenalpasti dalam biofilm yang terbentuk dari model *in situ* dan gigi yang terdiri daripada *Streptococcus, Neisseria, Haemophilus, Rothia, Prevotella, Fusobacterium, Veilonella, Actinomyces, Clostridium sensustricto dan Corynebacterium*. Dari segi tekanan kimia, gabungan SDF dan KI tidak mempunyai kesan anti-plak biofilm *in situ*, berbanding dengan SDF atau KI sahaja (p<0.05). Sebagai kesimpulan, sebuah model intraoral yang unggul dan mudah untuk mengkaji pembentukan biofilm *in situ* pada subjek manusia telah dihasilkan dan divalidasi dalam kajian ini. Dari segi klinikal, penggunaan tekanan kimia SDF dan KI, sama ada secara tunggal atau kombinasi, menghasilkan kesan biofilm anti-plak yang serupa.

DEVELOPMENT OF AN INTRAORAL DEVICE FOR *IN SITU* BIOFILM STUDY VALIDATED BY ITS EFFECTIVE APPLICATION IN ASSESSING BIOFILM CHARACTERISTICS

ABSTRACT

Modelling and characterising *in situ* oral biofilm development have played an important role, particularly in cariology, in understanding the pathogenesis and prevention of the disease, and new product development for caries control. However, the currently available models for this purpose are far from satisfactory. Hence, the objectives of this study, conducted in two phases, was first, to develop and validate an intraoral device for *in situ* biofilm development in human subjects, and second, to evaluate the effect of two different chemical stressors, silver diamine fluoride (SDF) and potassium iodide (KI), clinically used for caries control, on *in situ* plaque biofilms developed, using the validated device. In Phase one, a novel, acrylic, intra oral device carrying multiple transparent removable wells that could be comfortably worn by subjects was fabricated. To validate the device, an *in vivo* study was conducted to compare the microbial viability and composition between device-formed, and tooth-formed biofilms, using viability polymerase chain reaction (vPCR) and next generation sequencing (NGS) respectively. Structural characteristics of device-formed biofilm was analysed by confocal laser scanning microscopy (CLSM). In phase two, the device, thus validated, was utilized to compare the antibacterial efficacy of SDF and KI either singly or in combination, on device-formed, standardized, plaque biofilm. Multiple, device-formed *in situ* plaque biofilm samples were collected from six healthy subjects over 4 to 24 hr, for investigating various outcomes. The new device developed was economical, simple to fabricate, and permitted easy insertion

and removal by the subjects. Results showed no significant quantitative difference in viable bacteria between device-formed and tooth-formed biofilms ($p > 0.05$). The data revealed no significant difference between device-formed and tooth-formed plaque biofilms with regards to intraoral location of the device. However, comparison among subjects revealed significant differences in all viability parameters evaluated ($p < 0.05$). Furthermore, analysis of 24 hr *in situ* device-formed biofilm, under CLSM showed heterogenous biofilm configurations. On NGS analyses a total of 10 major bacterial genera were identified in both device-formed and tooth-formed biofilms and comprised: *Streptococcus, Neisseria, Haemophilus, Rothia, Prevotella, Fusobacterium, Veilonella, Actinomyces, Clostridium sensustricto* and *Corynebacterium*. In terms of chemical stressors, SDF and KI in combination had no significant anti-biofilm effect on *in situ* anti-plaque biofilm activity, compared with SDF or KI, alone ($p < 0.05$). To conclude, a novel, simple, intraoral device to study *in situ* biofilm development in human subjects was developed and validated. In clinical terms, the use of chemical stressors SDF and KI, either singly or in combination, appear to yield a similar anti-plaque biofilm effect.

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Intraoral devices for *in situ* oral biofilm development

The creation of *in vitro* biofilm models has contributed to significant advances in the study of biofilms including human dental biofilms (Azeredo *et al*., 2017). However, their known limitations have highlighted the need to develop *in situ* oral biofilm models. Such models include those based on an intraoral device that carries artificial substrates which allows undisturbed growth, isolation, and analysis of supra-gingival oral biofilms in its natural state. *In vivo* experiments that mimic natural oral conditions are inherently more complex but yield perhaps more realistic outcomes (Abdullah *et al*., 2019). These *in situ* models are required to enhance our knowledge on biofilm formation mechanisms, and to mitigate their contribution to oral diseases. Several factors can affect the development of *in situ* biofilm on artificial substrate such as the type of device and the substrate upon which the oral biofilms grow (Tomás *et al*., 2018).

Many intraoral devices have been developed for *in situ* oral biofilm development using different types of splints carrying a variety of substrates ranging from glass (Auschill *et al*., 2004), bovine enamel (Wood *et al.,* 2000) to hydroxyapatite (Takeshita *et al*., 2015; Xue *et al*., 2017). Although there are several *in situ* devices currently available to form supragingival *in situ* biofilm, they have several limitations including poor reproducibility and standardization (Prada-López *et al*., 2016).

1.2 Problem statement

The development of oral care products for prevention of oral diseases such as dental caries are mainly based on in vitro studies which can never mimic the actual natural oral environment. For this reason, results derived from in vitro studies should be interpret cautiously. Dental plaque biofilms are the prime movers of the most common oral pathologies such as dental caries and periodontal disease. It is therefore critically important to have a firm understanding of biofilm biology, and the first step in this direction is to study the colonisation profiles and the architecture of this complex community of organisms in its natural habitat *in situ.*

The currently available *in situ* models lack standardisation in biofilm development and in the way the biofilm was collected. In many models, the sample was collected from the tooth surface for analysis using paper points, cotton rolls, or scalers. These procedures potentially disturb the delicate three-dimensional relationship between cells, the extracellular matrix and the substrate, which directly influences the biofilm behaviour Therefore, "non-disturbing" biofilm methods must be applied especially in the study of antimicrobial agent, which means that the biofilm is not altered during its formation, collection, processing, or analysis. Furthermore, several other *in situ* devices require pre-treatment of teeth with etching for bonding and debonding of composite material for attachment of the substrate which could damage the enamel. Ideally, the device carrying the substrate should be able to retain in the mouth without being adhered to the tooth surface

1.3 Rationale of the study

To our knowledge, there is no standardised *in situ* biofilm collection device, and there is scant data on the relative superiority of one method over the other. Hence, there is an urgent need to develop and validate an intraoral device which is economical and simple to construct and at the same time allowing undisturbed growth and analysis of oral biofilm in its natural state. Such models would play an important role, particularly in cariology, from testing the effects of new caries prevention methods, to developing new caries-preventing products. Accordingly, a better knowledge of the characteristics of oral microbiome results in the development of better management strategies focusing on proactive management of oral health through an ecological approach to the host and its symbiotic microbial residents (Kilian *et al*., 2016).

No doubt the creation of *in vitro* models for biofilms has contributed significant breakthrough in the study of oral diseases, the limitations of these models have caused the scientific community to recognise that the *in vitro* models might not generate biofilms comparable to those found *in situ,* and therefore results must be interpreted cautiously. Hence, a validated *in situ* model could serve as an important tool for testing fundamental aspects of pharmaco-therapeutics in oral diseases such as in testing various antiplaque agents for caries prevention that could provide valuable scientific evidence in research for the oral health industry. For researchers, the output of this study will add value to the pool of scientific evidence in understanding biofilms as a major cause of dental diseases such as caries and periodontal diseases, and how the interaction of biofilms microbial populations affects bacterial virulence using quantitative and metagenomics analyses.

1.4 Objective of the study

1.4.1 General objective

To develop and evaluate an intraoral device for *in situ* oral biofilm development.

1.4.2 Specific objectives

1.4.2 (a) To develop and validate an intraoral device for *in situ* oral biofilm development.

i) To develop an intraoral device which allows undisturbed growth, collection, and analysis of *in situ* oral biofilms.

ii) To optimise the *in situ* biofilm formed on the intraoral device (device-formed biofilm) and supra-gingival biofilm grown naturally on the tooth surface (tooth-formed biofilm) at 4, 6, 12 and 24 hr.

iii) To compare the bacterial viability between device-formed biofilm and tooth-formed biofilms, among subjects and at different locations on the jaw.

iv) To identify the structural characteristics and thickness of 24 hr *in situ* device-formed biofilm using confocal laser scanning microscopy (CLSM).

v) To compare the bacterial composition between *in situ* device-formed and tooth-formed biofilm using next generation sequencing (NGS) technology.

1.4.2 (b) To assess the efficacy of newly developed intraoral device for *in situ* biofilm development in oral health research.

i) To determine the "working volume" for SDF

ii) To compare antibacterial efficacy of silver diamine fluoride (SDF) and silver diamine fluoride plus potassium iodide (KI) on 6 hr *in situ* biofilm.

1.5 Research questions

i) Is it possible to develop an intraoral device, which is simple to construct, reproducible and economical? Would this newly developed intraoral device allow undisturbed growth, isolation, and analysis of oral biofilm in its natural state?

ii) What are the qualitative growth characteristics of the *in situ* biofilm, in the newly developed device after 4, 6 12 and 24 hr?

iii) Is the bacterial viability on 24 hr device-formed and tooth-formed biofilm similar? Are they similar among the subjects and at different locations on the jaw?

iv) What is the structural architecture and thickness of 24 hr *in situ* device-formed biofilm viewed under CLSM?

v) Is the bacterial composition of device-formed and tooth-formed biofilm similar when analysed using NGS technology?

vi) What is the antibacterial efficacy of different volume of SDF in a 6 hr *in situ* biofilm?

vii) What is the antibacterial efficacy of silver diamine fluoride (SDF) and silver diamine fluoride plus potassium iodide (KI) combination in a 6 hr *in situ* biofilm?

1.6 Research hypotheses

i) It is possible to develop an intraoral device which is simple to construct, reproducible and economical and at the same time allowing undisturbed growth, isolation, and analysis of oral biofilm in its natural state.

ii) There will be an exponential growth of *in situ* biofilm in the newly developed device and on supragingival surfaces of natural teeth at 4, 6, 12 and 24 hr.

iii) The bacterial viability of 24 hr device-formed and tooth-formed biofilm is similar. They are similar among the subjects and at different locations on the jaw

iv) The structural architecture and thickness of 24 hr *in situ* device-formed biofilm can be viewed directly under CLSM.

v) The bacterial composition of *in situ* device-formed and tooth-formed biofilm is similar when analysed using NGS technology.

vi) The antibacterial effect of different volume of SDF on 6 hr *in situ* biofilm is similar.

vii) The antibacterial efficacy of silver diamine fluoride (SDF) and silver diamine fluoride plus potassium iodide (KI) on 6 hr *in situ* biofilm is similar.

1.7 Conceptual framework

Dental caries is an eventual end product of a dynamic interplay between pathologic factors that cause demineralisation and protective factors that lead to remineralisation of tooth enamel. Pathological factors include acidogenic bacteria, suppression of salivary protective function, and increased frequency of ingestion of fermentable carbohydrates; while protective factors include adequate salivary flow, numerous salivary components that foster commensal eubiotic bacterial growth, antibacterial agents (both natural and applied), fluoride from external sources, and a healthy diet. Dynamic balance between demineralisation and remineralisation of tooth enamel determines the eventual outcome of dental caries. The disease is reversible if detected early (Featherstone, 2004).

In health, there is an ecological balance between the human host and the indigenous microorganisms. Under healthy conditions, dental plaque plays an essential role in natural host defense mechanisms. Dental plaque is also the etiological agent associated with both dental caries and periodontitis and are frequently faced with factors that challenge a health-compatible state, including exposure to high sugar diet and tobacco smoke. Inefficient and insufficient oral hygiene measure, ageing processes and immune changes in the host can also affect the oral environment and can generate conditions that encourage the plaque microbiota to a disease-associated state (Marsh, 2018). In dysbiosis, diseases associated bacteria can grow to markedly higher proportions than under healthy condition, where they are normally minor and innocuous components in the biofilm (Marsh & Zaura, 2017).

In view of the role of oral microorganisms in the causation and pathogenesis of oral and systemic diseases, it is crucial to improve oral protection against pathogens and maintain dynamic equilibrium of the oral microecology. Our therapeutic goal is to re-establish its symbiotic equilibrium in whatever means that is appropriate in the individual patient such as by modulation of oral biofilms using various antiplaque agents. Figure 1.1 illustrates the interactions between various factors responsible for demineralisation/remineralisation of tooth structure in caries process and modulation of oral biofilms to maintain dynamic equilibrium of the oral microecology.

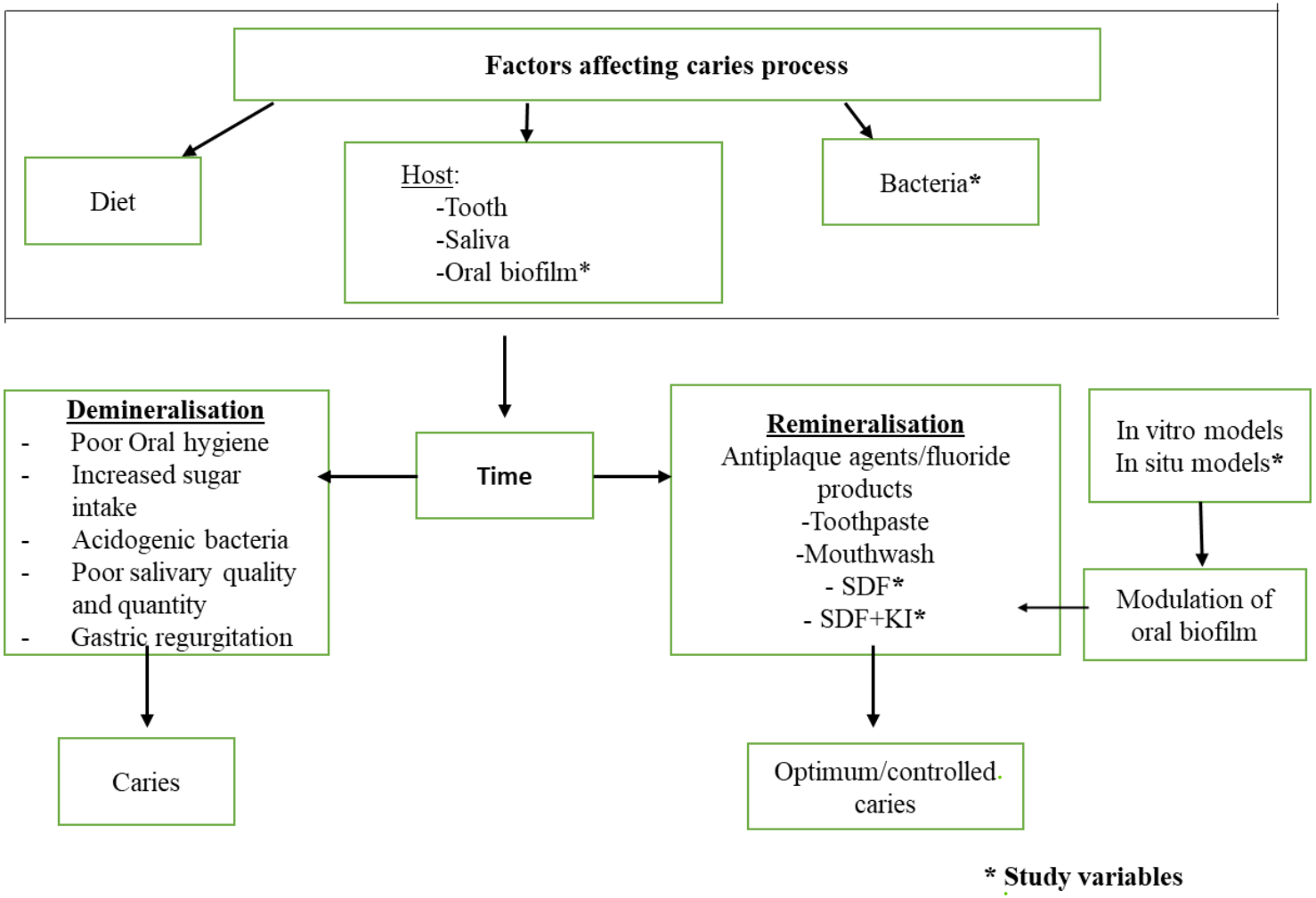


Figure 1.1 Conceptual Framework

CHAPTER 2

LITERATURE REVIEW

2.1 Oral biofilm

The history in the study of biofilm probably dated back to 1684, when Anthony Van Leewenhoek reported to the Royal Society of London "the number of animalcules in the scurf of a man's teeth is so many that I believe they exceed the number of men in the kingdom". In the mid-1800s, Robert Koch developed methods to create a solid nutrient medium to grow and isolate pure cultures of microorganisms. This development led to huge advances in the study of bacteria. Heukelekian & Heller (1940) revealed that surfaces enable bacteria to develop in substrates otherwise too dilute for growth, and that development takes place either as bacterial slime or colonial growth attached to surfaces. It was not until 1975 when the word "biofilm" first appears in scientific publication (Mack *et al*., 1975). Years later, in 1990 the United States National Science Foundation founded Biofilm Engineering research at Montana State University in Bozeman. Since then, the field of biofilm research has exploded. New tools and techniques are being explored to help in understanding the secrets of microbial diversity and community interactions within the biofilms. Attempts to characterise oral microbial diversity is progressively relying on cultivation-independent, molecular techniques (Hugenholtz 2002; Riesenfeld *et al*., 2004). Most of these molecular studies are based on the small subunit 16S ribosomal RNA (rRNA) gene because of its universal presence in cellular organisms, the presence of conserved regions, and its ability for phylogenetic analysis (Rajendhran & Gunasekaran, 2011). Bacteria are by far the predominant group of organisms in the oral cavity, with probably some 700 common oral species or phylotypes of which 50%-60% are

cultivable in the laboratory. Of these, approximately 54% are officially named, 14% unnamed (but cultivated) and 32% are known as uncultivable phylotypes (Samaranayake, 2018).

2.1.1 Development of oral biofilms

Biofilm development is a dynamic process comprising of several stages (Hall-Stoodley & Stoodley, 2002; Svensater & Bergenholtz, 2004; Kostakioti *et al*., 2013). The initial attachment of the bacterial cells is the critical stage for biofilm formation. Once attachment begins, depending on the environmental conditions, the bacteria can either progress to biofilm formation by adhering to the surface, or they can revert to the planktonic phase.

The first stage of biofilm formation involves the adsorption of macromolecules in the planktonic phase to the surface, leading to the formation of conditioning film or pellicle. All surfaces in the mouth are covered by a layer of adsorbed molecules of bacterial and of salivary origin (acquired pellicle), which is formed prior to the arrival of microorganisms and selectively promotes adhesion of certain microorganisms. When the planktonic microbe attaches itself to a surface, the organism can join with other microbes in the formation of a complex biofilm (Svensater & Bergenholtz, 2004).

The "pioneer" species coloniser are generally *Streptococci,* and as they grow, they modify the local environment and make conditions suitable for colonisation by more fastidious organisms (Samaranayake & Matsubara, 2017). After the establishment of initial colonisers, the next stage involves adhesion of microorganisms and attachment

may be strengthened through polymer production and unfolding of cell surface structures (Svensater & Bergenholtz, 2004), leading to the formation of distinct microcolonies after 4 to 24 hr. During this phase, the biofilm is not uniform in thickness, varying from sparsely colonised to almost full surface coverage. The biofilm grows basically by cell division, with the development of columnar microcolonies perpendicular to the tooth surface (Dige *et al*., 2007). The tooth surface is almost fully covered by microorganism within 24 hr. Secondary colonisers attach to receptors of already attached bacteria (co-adhesion). Continuous adsorption of planktonic microbes from saliva, in addition to cell division, contributes to the expansion of the biofilm. In the surface layer, coaggregation of different species creates "corncorb" structures. The biofilms become thicker between 24 and 48 hr (Samaranayake & Matsubara, 2017).

Figure 2.1 illustrates stages of biofilm formation:

1) Reversible attachment of the planktonic cells (brown ovals), followed by the adhesion to the surface (grey),
2) The bacteria then form a monolayer and irreversibly attach by producing an extracellular matrix,
3) A microcolony is formed, multilayers appear, later on mature forming "mushroom" like characteristics,
4) Some cells start to detach and,
5) The biofilms in the planktonic phase disperse.

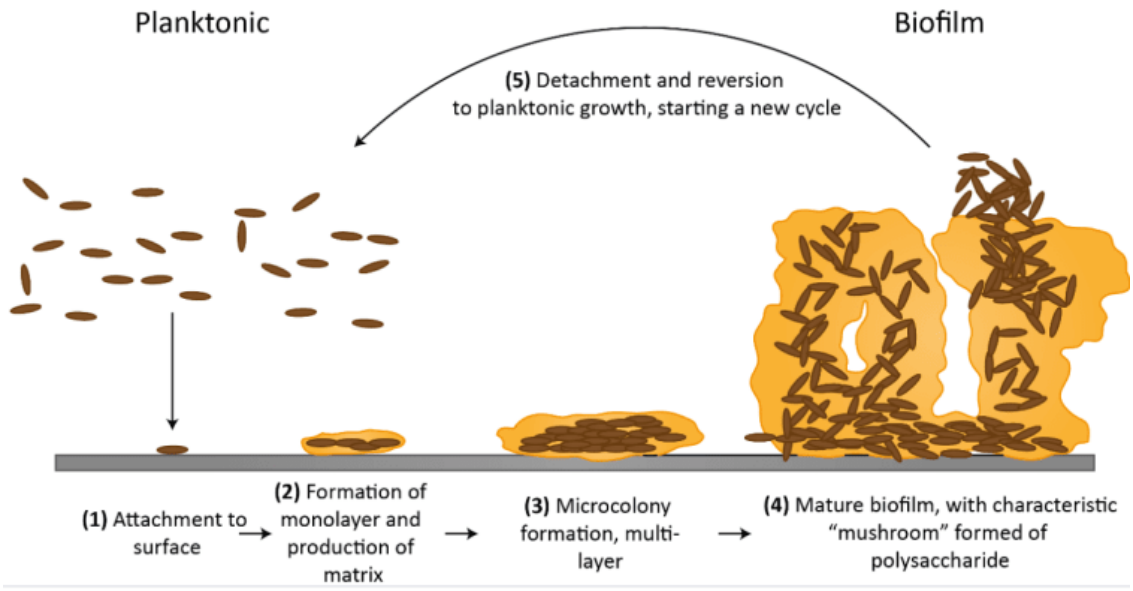


Figure 2.1 Schematic representation of biofilm formation (Adapted from Vasudevan, 2014)

As the biofilm develops, the metabolism of the initial colonisers modifies the environment in the developing biofilm, creating a local condition that is either more attractive to later colonisers or becoming more unfavorable for the pioneer group, for example, by making it more anaerobic after their consumption of oxygen or accumulating inhibitory metabolic products (Marsh, 2010). These environmental changes in the biofilm lead to a gradual replacement of the initial colonisers by other bacteria more suited to the modified habitat; this process is termed microbial succession which takes from one to seven days. This sequence of events gradually increases the species diversity of the biofilm in the dental plaque, concomitant with continued growth of microcolonies (Samaranayake & Matsubara, 2017).

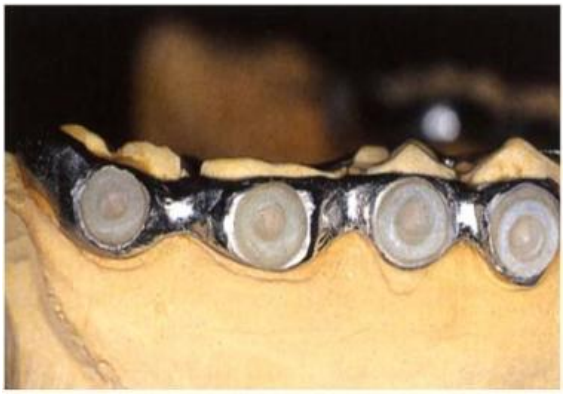
A shift from aerobic and facultative anaerobic species (mainly *Streptococcus*) was observed in the early stage of biofilm to facultatively and obligatory anaerobic organisms, gram-negative cocci and rods, fusobacteria, spirochetes, and actinobacteria (especially *Actinomyces*) after nine days. A mature biofilm can be found after one week or more until it reaches a critical mass after which some bacteria may detach and enter the planktonic phase and be transported to new colonisation sites, thus restarting the whole cycle (Samaranayake & Matsubara, 2017).

The rate of detachment of microorganisms from the dental biofilms is not clear. It is seen as a continuous process during development, and localised detachment of microorganisms is likely to start after initial adhesion and increase with time as it is related to the number of microorganisms present in the biofilms (Svensater & Bergenholtz, 2004). The fact that microorganisms detach regularly has implications for their spreading and colonisation to other sites.

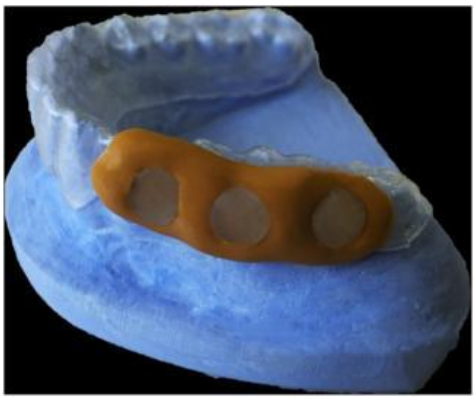
Over the last decades, numerous *in vitro* and *in vivo* studies of oral biofilms have significantly contributed to our present knowledge of biofilm formation and how the sophisticated microbial community interactions take place within the biofilms (Bowden & Hamilton, 1998; Marsh, 2004)**.** The oral biofilms function as microbial community and collectively display properties that favor their formation and persistence in the oral cavity. The bacteria interact, both synergistically and antagonistically. They combine forces to breakdown complex host macromolecules to obtain nutrients. Food chain develops, cell-cell signaling occurs which facilitates the coordination of gene expression among members in the microbial community. Thus, oral biofilms become structurally and functionally organised (Zijnge *et al*., 2010) and their biological properties are greater than the sum of the individual species (Kilian *et al*., 2016.). These biofilms are also more tolerant to antimicrobial agents and host defenses (Do *et al*., 2013).

2.1.2 The intraoral device for development of *in situ* oral biofilm growth

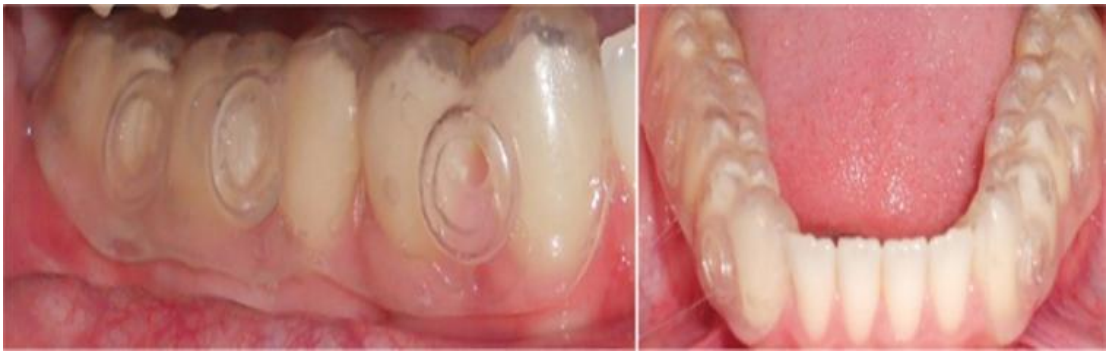
The intraoral devices used for development of *in situ* oral biofilm has evolved rapidly that resulted in many of these devices are left without standardisation or control. A well-designed device with proper validation is important to ensure device-formed biofilms has similar characteristics to tooth-formed biofilm. Researchers have tried to design different types of intraoral devices to grow natural biofilms (Figure 2.2). Some used the subject's own prosthesis such as denture (Ostrom & Koulourides, 1976; Thuy *et al*., 2008) and orthodontic appliances (Ostrom & Koulourides, 1976; Jongsma *et al*., 2015).



a) A metal device (Simion *et al*., 1997)



b) Thermoplastic polysilicone splint (Sennhenn-Kirchner *et al*., 2007)



c) The intraoral device of overlaid- holding splint IDOD (Prada-López *et al*., 2015b)

Figure 2.2 Different types of intraoral devices designed to grow natural biofilms.

Abdullah *et al.* (2019) conducted a systematic review to identify all studies using intraoral devices for collection of *in situ* oral biofilms for microbiological analyses. They found that, given the plethora of methods, substrates, and subjects/cohorts used by different researchers reviewed, it was extremely difficult to state whether one method is superior to another, and hence no uniformly superior method of collecting *in situ* biofilm has emerged. Nevertheless, their review of the methodology, should assist the novice in selecting the best method for his/her own experimental needs for *in situ* biofilm collection. However, in terms of the analysis of the *in situ* microbiome/microbiota, they concluded that next generation sequencing (NGS) and the rapidly emerging, high fidelity, so-called `third generation` sequencing techniques will be the future.

Table 2.1 shows characteristics of study subjects, intraoral device, characteristics of the substrates, biofilm age, methods used to study *in situ* oral biofilm and outcome measures as identified in their reviews.

Table 2.1 Characteristics of study subjects, intraoral device, substrates, and methods used to study *in situ* oral biofilm

Author. Year*	Number of subjects (age group in years)	Device		Substrate					Device-formed oral biofilm		
		Material used	Location	Type	Location	Number	Shape	Size	Age	Outcome measures	Methods of analysis
Wood *et al*. (2000)	8 (not stated)	Nylon (Leeds *in situ*)	Upper jaw	Human enamel	Buccal	2	Not stated	Not stated	4 days	Architecture	CLSM
Giertsen *et al*. (2000)	11 (21-28)	Acrylic	Lower jaw	Bovine enamel	Buccal	2	Cylindrical	6.8 x 1.5mm	7 days	Total cell count viability,	Culture, immunofluorescence
Wood *et al*. (2002)	4 (not stated)	Nylon (Leeds *in situ*)	Upper jaw	Human enamel	Buccal	2	Not stated	Not stated	2,7,14 and 28 days	Architecture thickness	CLSM
Auschil *et al*. (2004)	8 (23-30)	Acrylic	Upper lower jaw, palatal	Glass	Buccal (upper, lower jaw) and palatal	9 -upper appliance, 6 - lower appliance	Cylindrical	3x2mm	48 hr	Thickness	CLSM
Auschil *et al*. (2005)	7 (25-29)	Acrylic	Upper jaw	Glass	Buccal	6	Cylindrical	3 x 2 mm	48 hr	Thickness, vitality	CLSM
Dige *et al*. (2007)	10 (21-35)	Acrylic	Lower jaw	Glass	Buccal	Not stated	Cuboidal	4x4x1mm	6,12,24 and 48 hr	Structure, composition	FISH, CLSM
Al-Ahmad *et al*. (2007)	1 (27)	Acrylic	Upper jaw	Bovine enamel	Buccal	6	Cylindrical	3x2mm	1,2,3,5,7days	Thickness, composition	FISH, CLSM
Dige *et al*. (2009)	10 (23-36)	Acrylic	Upper jaw	Glass	Buccal	6	Cuboidal	4x4x1mm	6 hr,12 hr,1 and 2 days	Quantification of bacteria	FISH, CLSM
Al-Ahmad *et al*. (2009)	6 (not stated)	Thermoplastic	Upper jaw	Bovine enamel	Buccal	6	Cylindrical	5x1.5mm	2,6 and 12 hr	Adherence of bacteria to device	FISH, TEM, SEM
Jung *et al*. (2010)	6 (not stated)	Thermoplastic	Upper jaw	Bovine dentine	Buccal	6	Cylindrical	5x1.5mm	30 mins, 2 and 6 hr	Total bacterial count, adhesion to substrate	Culture, FISH, CLSM, SEM, TEM

Table 2.1 Characteristics of study subjects, intraoral device, substrates, and methods used to study *in situ* oral biofilms (cont.)

Author. Year*	Number of subjects (age group in years)	Device		Substrate					Device-formed oral biofilm		
		Material used	Location	Type	Location	Number	Shape	Size	Age	Outcome measures	Methods of analysis
Gu *et al*. (2012)	9 (25-42)	Acrylic	Upper jaw	Glass	Buccal	6	Cylindrical	3x1.5mm	48 hr	Thickness, vitality	CLSM
Tawakoli *et al*. (2013)	6 (not stated)	Not stated	Upper jaw	Bovine enamel	Buccal	6	Cylindrical	5x1.5mm	2 hr	Vitality, adherence to substrate	Culture, florescence microscope, TEM
Langfeldt *et al*. (2014)	32 (20-30)	Acrylic	Upper & lower jaw	Membrane filters	Buccal	8	Not stated	Not stated	1.3.5,9 and 14 days	Composition	DNA sequencing
Takeshita *et al*. (2015)	19 (20-28)	Acrylic	Lower jaw	HA	Buccal	6	Cylindrical	5mm	1,2,3,4,5 and 7 days	Composition	Real-time PCR, DNA sequencing
Prada-López *et al*. (2015a)	5 (20-45)	Inner: EVA Copolymers Outer: Polyethylene terephthalate (IDODS)	Lower jaw	Glass	Buccal	6	Not stated	5mm	2 hr	Thickness, vitality, architecture	CLSM
Quintas *et al*. (2015)	15 (20-30)	IDODS	Lower jaw	Glass	Buccal	6	Not stated	6x1mm	2 and 4 days	Thickness, vitality, covering grade	CLSM
Prada-López *et al*. (2015b)	20 (20-45)	IDODS	Lower jaw	Glass	Buccal	6	Cylindrical	6x1mm	2 and 4 days	Vitality, structure, covering grade	SEM, CLSM
Dige *et al*. (2016)	10 (22-36)	Acrylic	Lower jaw	Glass	Buccal	8	Cylindrical	4x4x1mm	2 and 4 days	Extracellular pH	CLSM

Table 2.1 Characteristics of study subjects, intraoral device, substrates, and methods used to study *in situ* oral biofilms (cont.)

Author. Year*	Number of subjects (age group in years)	Device		Substrate					Device-formed oral biofilm		
		Material used	Location	Type	Location	Number	Shape	Size	Age	Outcome measures	Methods of analysis
Wake *et al*. (2016)	10 (26-30)	Acrylic	Upper jaw	HA	Buccal	8	Cylindrical	6x1.5mm	1,4,8,12,16,24,48,60,72 and 96 hr	Thickness, viability, composition	Culture, Real-time PCR, CLSM, SEM, TEM, DNA sequencing
Klug *et al*. (2016)	25 (20-25)	Acrylic	Upper jaw	Human enamel dentine	Buccal	6	Cylindrical	6x4mm	48 hr	Vitality, structure, composition	CLSM, FISH, DNA sequencing
Tawakoli *et al*. (2017)	9 (21-41)	Acrylic	Lower jaw	Glass	Buccal	Not stated	Cuboidal	4x4x1mm	48 hr	Spatial distribution, composition	CLSM, DNA sequencing
Xue *et al*. (2017)	12 (mean 22.5±2.6)	Not stated	Upper jaw	HA	Palatal	6	Cuboidal	4x4x2mm	2 weeks	Lactic acid, vitality, biomass	SEM, CLSM, MTT assay
Quintas *et al*. (2017)	18 (20-45)	IDODS	Lower jaw	Glass	Buccal	6	Cylindrical	6x1mm	48 hr	Thickness, vitality, covering grade	CLSM
Tomas *et al*. (2018)	15 (20-45)	IDODS	Upper and lower jaw	Human enamel, HA, glass	Buccal	6	Cylindrical	7x2 mm	48 hr	Thickness, vitality, composition	CLSM, DNA sequencing

*studies arranged in chronological order
CLSM used following staining with proper live/dead fluorochromes
CLSM: Confocal Laser Scanning Microscopy, FISH: Fluoresent *in situ* Hybridisation, TEM: Transmission Electron Microscopy, SEM: Scanning Electron Microscopy, PCR: Polymerase chain reaction, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

2.1.2 (a) The devices and substrates used to collect biofilms.

Table 2.1 showed that most researchers in previous studies constructed the intraoral devices on either the maxillary or mandibular jaw model of the subjects. However, Auschill *et al*. (2004) and Tomás *et al*. (2018), placed the device on both jaws to compare the characteristics of *in situ* biofilms from both jaws.

The substrate used in previous studies varies from human derived enamel in the "Leeds *in situ* device" (Wood *et al*., 2000; 2002), human or bovine enamel-dentine slab, glass disks or hydroxyapatite disks. The glass disks substrates were manufactured to custom made for certain size, non-fluorescent, and with a surface roughness of 1,200 grit. When the human/dentine slabs were used, they were prepared from extracted human teeth and sterilised before cutting, grinding and polished according to shape and size needed in the study. Hydroxyapatite has been used in some studies to allow growth of bacteria on synthetic media mimicking dental tissues, thereby avoiding the use of extracted teeth. The number of substrates placed in the previous experiments varies from two (Giertsen *et al*., 2000; Wood *et al*., 2000; 2002) to eight (Wake *et al*., 2016). Most workers used six substrates on the buccal side of the device, by placing three substrates on each side of the jaw. The shape of the substrates used in the study were only either cylindrical or square shape and their sizes vary. For the cylindrical disks, the size mostly used were with diameter varying from 3 mm by most workers to 7 mm (Tomas *et al*., 2018), while their height varies from 1 to 2 mm (Auschill *et al*., 2004; 2005; Quintas *et al*., 2015; 2017; Tawakoli *et al*., 2017). It was found that acrylic material has been the material of choice, however, Wood *et al*. (2000; 2002) used "Leeds *in situ* device", which composed of a nylon ring to hold an enamel substrate, a technique which was previously been described by Robinson *et al*. (1997). Prada-

López *et al*. (2015b) developed and patented the Intraoral Device of Overlaid Disk-holding Splint (IDOD) for *in situ* biofilm development.

2.1.2 (b) Subjects and biofilm age

As shown in Table 2.1, subject recruitment in the previous studies include healthy subjects with age range from 20 to 45 years old. They were among dental students or staff from the medical or dental faculty, the number of subject recruited ranges from one (Al-Ahmad *et al*., 2007) to 25 (Klug *et al*., 2016). The inclusion and exclusion criteria employed were similar in most studies. Participants who were systemically healthy with good oral health, having a minimum of 24 permanent teeth present in the mouth, with no evidence of gingivitis or periodontitis and an absence of untreated caries were included in the study. Several studies excluded smokers, those wearing dental prostheses or orthodontic appliance, on antibiotic treatment or those who use oral antiseptics routinely in the past three or six months. Some researchers included both male and female subjects in their study. However, Klug *et al*. (2016) included only male subjects, while other studies did not mention gender of the subjects enrolled. Tawakoli *et al*. (2017) however have excluded pregnant and breastfeeding women in their study.

The duration of *in situ* biofilm grown for the study varies from 30 min (Jung *et al*., 2010) to 28 days (Wood *et al*., 2002). Some studies collected the biofilm at one time point only; for example, after two days, four days or seven days; while other studies grew the oral biofilm for a duration of time and collection was done at specific time point along the study; for example, Dige *et al*. (2007) collected the biofilm after 6,

12, 24 and 48 hr. Variation in biofilm age and frequency of biofilm collection in the studies were related to the purpose of the investigation.

2.1.2 (c) Characteristics of oral biofilm following exposure to chemical agents

In translational terms, the *in situ* biofilm model once standardised and calibrated should be ideal for evaluating the effect of chemical agents on biofilm microbiota. The review identified several researchers who have embarked into the study looking at the ecological changes of biofilms exposed to various antimicrobial agents (Table 2.2). Studies on biofilms not exposed to chemical agents are listed in Table 2.3. The main outcome measures evaluated in the latter studies were bacterial viability (live/dead ratio) and bacterial biomass or thickness of the biofilm, analysed using CLSM.

As shown in Table 2.2, several studies investigated the effect of chemical agents on *in situ* biofilm. The main chemical agents used in the previous studies were chlorhexidine gluconate, amine fluoride/stannous fluoride, zinc chloride, alcohol, and essential oil. It was noted that both chlorhexidine and amine fluoride/stannous fluoride significantly reduced the biofilm thickness and biofilm viability compared to controls, but the differences between the two agents were not significant (Auschill *et al.,* 2005). In another study, Gu *et al*. (2012) evaluated the use of zinc chloride at 2.5, 5, 10 and 20 mM concentrations, and results showed significant reduction in the plaque index, biofilm thickness and biofilm viability compared with the controls. They also evaluated the effect of zinc chloride on various biofilm layers and reported that 2.5 mM was the lowest concentration to inhibit the outer layer, 5 mM was the lowest to inhibit the middle layer, while none of the zinc chloride concentrations could inhibit the bacteria in the inner layer.