

Review Article

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Oral Biofilms

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Biofilms are the form of life for great majority of oral bacteria and contribute to various oral diseases. To maintain oral health a balance between the human host and the microbes is essential. The excessive formation of oral biofilm results in intraoral diseases such as dental caries, gingivitis and periodontitis. The ability of pathogenic bacteria to form a biofilm increase their resistance to antibiotics. The most commonly pathogenic bacteria found in oral biofilms are methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Streptococcus mutans*. The molecular understanding of the formation and organisation of oral biofilms has just begun to emerge. The chapter focuses on oral pathobionts as commensals of oral biofilms and how they cause disease and various methods to study these oral biofilms.

Introduction

Oral cavity

The oral microbes comprise a complex community and that oral health or disease depends on the interface between the host and the microbial community. The oral ecosystem harbours more than 700 bacterial taxa. The bacteria colonize the hard surface of the teeth and the soft tissue of the oral mucosa. Different bacterial flora exists in the teeth, gingival sulcus, tongue, cheeks, hard and soft palate and tonsils (Dewhirst *et al.*, 2010). The major genera found in the healthy oral cavity include *Streptococcus*, *Veillonella*, *Granulicatella*, *Gemella*, *Actinomyces*, *Corynebacterium*, *Rothia*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*,

Neisseria, *Haemophilus*, *Treponema*, *Lactobacterium*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, *Eubacterium* and *Propionibacterium* (Wilson, 2005; Zaura *et al.*, 2009; Em *et al.*, 2010). Although the microbiome differs between the individuals, the microbiome per individual is constantly same in time (Badger *et al.*, 2011).

Development of oral biofilm

Most of the oral bacteria are found in the oral biofilm which remains stable despite environmental changes (Marsh and Bradshaw, 1995). However, under certain conditions shifts in the composition of the dental plaque biofilm could lead to dental diseases such as dental caries and periodontal diseases. The bacterial composition in biofilm from a

healthy peridontium is different from that of periodontitis (Lamont and Hajishengallis, 2014). The commensal bacteria in the mouth inhibit the colonization of pathogenic bacteria (Wade, 2013). However, the disruption in commensal biofilm results in the adherence and accumulation of pathogens in oral surfaces. The microbes adhere to the oral cavity and initiate the development of dental plaque biofilm. A highly organised sequence of events occurs. First, the adhesion of the microorganism on the surface of oral cavity takes place. The development of new plaque starts as soon as the tooth surface is covered with the protein film (pellicle). This process is very fast and takes place in minutes to form, after eruption of a new tooth or after removal of previous plaque (Hannig, 1999). The pellicle comprises of several molecules derived from the host and mainly contains mucins, proteins, agglutinins. Secondly, the multiplication of bacteria takes place.

The “early colonizers” bind to the pellicle covered tooth surface and mainly include streptococci and several gram-positive rods (Marloes *et al.*, 2017). The early colonizers are then replaced by the “late colonizers”. The biofilm is now dominated by Gram negative anaerobic bacteria. *Fusobacterium nucleatum* co-aggregates with other bacteria bridging organism by binding streptococci to pathogens such as *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia* and *Tannerella forsythia* (Kolenbrander *et al.*, 2010).

Thirdly, the micro-colonies formed start to express a biofilm phenotype and excrete extracellular polymeric substance (EPS) leading to formation of three dimensionally spatially arranged mature biofilm community (Kolenbrander *et al.*, 2002). The EPS present in the biofilm protects it from the mechanical flow of the saliva and other forces in the oral cavity (Stephens, 2002).

Bacterial interaction in the oral biofilm

The initial flora within few hours after birth is primarily *Staphylococcus epidermidis* and Streptococcus species mainly *S. salivarius* (Nelsen-Filho *et al.*, 2013). The mature oral microbiome consists of hundreds of bacterial species. An ecologically balanced diverse microbiome which is in equilibrium with the host predominantly consists of Gram-positive facultative anaerobic bacteria, such as the *Streptococcus* and *Actinomyces* genera predominantly in healthy individuals (Jiao *et al.*, 2014). Different studies have suggested a core oral microbiome of health (Fig. 1) which is different from the diseased one. A study done by Bik *et al.*, (2010) on the largest set of near full length sequences in healthy individuals analysed 10 variables shared between 11 bacterial species. This supported the concept of both core and variable microbiome within the oral cavity.

The major genera in the healthy oral cavity includes *Streptococcus*, *Veillonella*, *Granulicatella*, *Gemella*, *Actinomyces*, *Corynebacterium*, *Rothia*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Neisseria*, *Haemophilus*, *Treponema*, *Lactobacterium*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, *Eubacterium* and *Propionibacterium* (Dewhirst *et al.*, 2010). Recently a study by (Mark Welch *et al.*, 2016) utilizing combinational labelling and spectral imaging FISH has observed an oral microbiome termed as hedgehog structure consisting of nine taxa arranged in an organised spatial framework, which includes *Corynebacterium*, *Streptococcus*, *Porphyromonas*, *Haemophilus* / *Aggregatibacter*, *Neisseriaceae*, *Fusobacterium*, *Leptotrichia*, *Capnocytophaga* and *Actinomyces*. This hedgehog structure mainly consists of *Corynebacterium* as a multitaxon filament rich annulus and peripheral corn-cob structure.

These filaments are comprised of *Fusobacterium*, *Leptotrichia* and *Capnocytophaga* whereas they are surrounded by *Streptococcus*, *Porphyromonas* and *Aggregatibacter*. *Corynebacterium* organisms are considered to be bridge species in regard to biofilm formation. Thus the oral biofilm is a network of multiple synergistic and antagonistic interactions generating microbial interdependencies and give biofilms a resistance from minor environmental disturbances. If these environmental disturbances exceed the threshold associated with health, a dysbiosis occurs between microbes causing the oral diseases (Marsh and Zaura, 2017).

Distribution of genomes sequenced per phylum

Biofilm in dental caries

Frequent intake of dietary sugars results in proliferation of bacteria such as *Streptococcus mutans* and *Lactobacillus acidophilus* creating an acidic environment causing the demineralisation of the enamel of the tooth (Marsh, 2010). The non-cariogenic plaque changes to cariogenic plaque causing the development of dental caries. *Streptococcus sanguinis* decreases after a micro-ecological imbalance being one of the causes resulting in dental caries. Other studies have shown that many other bacterial cariogenic species like *Streptococcus mitis*, *Rothia*, *Actinomyces*, *Lactobacillus*, *Bifidobacterium* and even fungal species like *Candida albicans* can account for a biofilm becoming cariogenic (Klinke *et al.*, 2011). Recently a new cariogenic bacteria *Scardovia wiggsiae* has been found to be associated with dental caries (Tanner *et al.*, 2011). The ecological changes which these organisms bring in the biofilm changing it to cariogenic by down regulating the main energy generating glycolytic pathway in order to be acid tolerant and also

some bacteria undergo phenotypic changes in health and disease.

Biofilm in periodontal disease

Periodontal biofilms are formed by co-aggregation of opportunistic microorganisms caused by diverse factors like poor oral hygiene. The periodontal tissue inflammation in the early stages is known as gingivitis and can be treated by removing the dental biofilm. The presence of dental plaques in gingival tissue and interactions of pathogens with host cells cause inflammation leading to periodontitis. Chronic periodontitis is now linked to systemic inflammatory diseases like atherosclerosis, infective endocarditis, diabetes, respiratory diseases and rheumatoid arthritis (RA) (Kim and Amar, 2006; Gaffen *et al.*, 2014; Hajishengallis, 2015). In periodontitis there is proliferation of gram negative pathogenic bacteria like *P.gingivalis*, Spirochaetes like *Treponema denticola* in the dental plaque biofilm (Pihlshrom *et al.*, 2005). The three bacterial species of the “red complex” *Tanerella forsythia*, *P. gingivalis*, *T. denticola* are the indicators of the periodontitis. A small rise in pH allows the periodontal pathogens to overgrow other microorganisms in the dental plaque (Marsh, 1994). Factors like smoking contribute to enhancement of colonisation of periodontal pathogens like *Tanerella forsythia*, *P.gingivalis*, *T.denticola*, *P.intermedia*, *Parvimonas micra*, *Prevotella nigrescens* and *Campylobacter rectus* (Shchipkova *et al.*, 2010). Recent studies have shown that bacteria like *Selenomonas* have association with periodontal disease (Drescher *et al.*, 2010) (Table 1).

Diagnostic techniques to study oral biofilms

The currently available models and techniques to study oral biofilms include: Saliva- Saliva only or its combination with selective media

can be used. In multispecies biofilms the medium described by (Guggenheim *et al.*, 2001) can be used. To this fluid universal medium (FUM) can be added like N-acetyl Muramic acid for *T. forsythia*; 0.34 mM hemin for *P. gingivalis* (Ammann *et al.*, 2013).

Support surfaces to study Oral Biofilm

Inert surfaces like glass tube containing specific medium and Sucrose 1%.

The cultures were incubated at 37 °C with an angle of 30 degrees. Biofilm formation was evaluated after 24-48 hours with 0 for no adhesion and 4 to strongly adhesive (Murchison *et al.*, 1981). However this surface allows to quickly screen the biofilm formation ability of some strains only.

Dentin-Dentin discs both human and bovine origin have been used (Li *et al.*, 2014)

Enamel- Enamel is mostly used substratum for cariogenic biofilm models. It may be of human or bovine origin (Van de Sande *et al.*, 2011).

Polystyrene- Polystyrene microtiter plates provide a sterile abiotic surface for studying bacterial formation. The 96 well microtiter plate is used to study biofilms.

Hydroxyapatite- Saliva coated hydroxyapatite beads have been used in various studies. Dual biofilms of *Streptococcus sobrinus*, *S.mutans* and *S.sanguinis*, *S.mutans* and *Veilonela parvula* strains have been studied using this model (Kara *et al.*, 2006; Rozen *et al.*, 2001; Li *et al.*, 2014).

Non cultivable methods to study oral Biofilms

In Situ Hybridization Fluorescence (FISH).

Multiple species of microbes can be detected in biofilm sample using FISH (Thurnheer *et al.*, 2004). It is possible to perform several consecutive FISH procedures with multiple rRNA to identify simultaneously many members of biofilm (Almstrand *et al.*, 2013).

Confocal Laser Scanning Microscopy (CLSM)

CLSM enables to observe the biofilms in 3 dimensions. High quality biofilm images are collected and visualised. Briefly a 15 minutes dark incubation period is given, an excitation wavelength of 488nm to collect all light emitted between 500 and 550 nm and over 560nm by various filters is used. The scan mode time series of time lapse scans at intervals of 10 s during 590 s in continuous scanning mode with 10 X objective lens is then used (De *et al.*, 2012). The distribution of live and dead cells at the different biofilm development times can also be investigated by this method.

Scanning Electron Microscopy (SEM)

In scanning electron microscopy biofilms are fixed for 24 hours in a 2.5% glutaraldehyde solution and dehydrated with a graded ethanol series, subjected to a critical point drying with CO₂, covered with gold (10nm) and examined under SEM. SEM has been used to study the role of red complex bacteria in the colonization of gingival epithelia by sub gingival biofilms, multispecies biofilms have been studied by this method (Standar *et al.*, 2010; Thumheer *et al.*, 2014)

Polymerase Chain Reaction (PCR)

PCR is the most conventionally used molecular biology approach to identify and count bacterial species in oral biofilms in oral diseases like caries and periodontitis. PCR based methods have been used in single or

multiplex approaches which involve amplification and sequencing of fragments of the 16S rRNA gene to detect bacterial species associated with periodontal disease or caries (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (Elick and Pfister, 2002; Garcia *et al.*, 1998; Rocas *et al.*, 2001). Quantitative real-time PCR is used to observe microbial shifts in caries and periodontitis models (Zaura *et al.*, 2011; Karched *et al.*, 2015). However the limitation of the q PCR is its inability to discriminate between live and dead cells.

Checkerboard DNA-DNA hybridization

New molecular methods include application of DNA probes specific to oral bacterial species in checkerboard DNA- DNA hybridization, which initially allowed the detection and enumeration of bacteria species from around 43 biofilm samples simultaneously (Socransky *et al.*, 1994). Using this technique around 40 taxa in supragingival and subgingival plaque samples it was found that *Actinomyces* species is the most established species at oral sites (Ximenz-Fyvie *et al.*, 2000).

Table.1 The diversity of human oral microbiome (Human Oral Microbiome Database website, www.homd.org)

Phylum	Number of genomes in the phylum	Number of taxa in the phylum	Number of taxa with genomes
<i>Actinobacteria</i>	222	118	94
<i>Bacteroidetes</i>	135	125	63
<i>Chlamydiae</i>	5	1	1
<i>Chlorobi</i>	0	3	0
<i>Chloroflexi</i>	4	3	1
<i>Cyanobacteria</i>	1	1	1
<i>Euryarchaeota</i>	1	1	1
<i>Firmicutes</i>	699	266	167
<i>Fusobacteria</i>	54	37	22
<i>Gracilibacteria</i> (GN02)	2	5	5
<i>Proteobacteria</i>	434	141	89
SR1	1	5	1
<i>Saccharibacteria</i> (TM7)	7	19	5
<i>Spirochaetes</i>	35	50	8
<i>Synergistetes</i>	8	8	3
WPS-2	0	1	0

16S rRNA PCR cloning methodology

Because not all microbes can grow in culture medium, a culture independent technique started with the implementation of 16S rRNA PCR cloning sequencing methodology amplifying the 16S rRNA genes directly from samples. Amplicons were then cloned and sequenced. Many studies have used this

technique to identify oral microbiota profiles in health and disease (Riggio *et al.*, 2008; Tanner *et al.*, 2011). This has led to the development of Human oral microbiome database (Human Oral Microbiome Database, 2012) which aims to identify and catalogue all the species found in oral cavity. More rapid identification of human oral bacterial 16S rRNA gene sequences as compared to other

methods has been used recently in CORE 16S rRNA DNA database (Griffen *et al.*, 2011).

Next generation sequencing methods

The next generation sequencing methods are based on the detection of pyrophosphate released during DNA synthesis. The DNA libraries are constructed from the sample and the DNA fragments are amplified within an emulsion and sequencing done. The nucleotide sequence data is then read (Ronaghi, 2001). The data is then processed through bioinformatics, compared with sequences from available databases like Human Oral Microbiome database or the ribosomal database project (Cole *et al.*, 2009).

Many studies of oral biofilm have been done using this method. Saber *et al.*, (2012) have seen that *Streptococci*, *Actinomyces* and unknown taxa are present in the microbiota of symptomatic periapical lesions. Another study by Ahn *et al.*, (2011) determining the oral microbiome profile in oral cancer volunteers showed the presence of *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Acinobacteria* and *Fusobacteria*. Pyrosequencing provided better sensitivity in identification of the bacteria.

The drawback of this technique is that the sequencing errors result in overestimation of microbial diversity and closely related species cannot be differentiated. The basic principles of biofilm formation are now known but the molecular understanding of the formation and structural organisation of various types of biofilms has just begun to emerge (Bjarnsholt *et al.*, 2018).

Limitations of molecular analysis of oral biofilms

Molecular methods of analysis of oral biofilms are expensive, time consuming and

require high level of expertise to process the data. The inability of this technique to use in clinical setting limits their use in diagnostics. The polymicrobial nature of oral diseases limits the use of oral biofilms to be used in chairside tests as diagnostic tools. However, they have a potential in, “minimally invasive dentistry”.

Control of pathogenic bacteria in the oral biofilm

The pathogen associated biofilms can evade human host defences and are frequently associated with persistent infections, resistant to antibiotic therapy (Kostakioti *et al.*, 2013). The concentrations of antibiotics needed to eradicate a biofilm are in the range of 100-1000 times more than minimum inhibitory concentration (MIC) (Hoiby *et al.*, 2010). A combination of antimicrobial peptides and essential oils, biofilm degrading enzymes, quorum sensing inhibitors and nanoparticles as potential biofilm agents have been used (Algburi *et al.*, 2017). Recently bacteriocins which are antimicrobial peptides produced by bacteria typically 2-10KDa in size generally targeting closely related bacteria particularly the class I bacteriocin known as lantibiotics are being used (Mathur *et al.*, 2018). *S.mutans* is an important organism causing dental caries. Several studies have been attempted to target *S.mutans* to see effect of various reagents on *S.mutans* biofilms (Clark *et al.*, 2017). Antibiofilm activity against *Enterococcus faecalis* another biofilm forming organism in the oral cavity has also been attempted. *E.faecalis* biofilms have reduced levels of extracellular polysaccharides as compared to *S.mutans* biofilms. *Actinomyces viscosus* is frequently found as a biofilm former in the periodontal pockets (Balto *et al.*, 2015). Like *E.faecalis*, it is also implicated in endocarditis. Studies have been reported to control the biofilm of this organism. *Candida albicans* biofilms

associated with oral thrush have also been targeted for disruption of hyphal formation essential for *C. albicans* biofilm formation (Graham *et al.*, 2017).

The oral microbiome is diverse with multispecies microbial communities in oral surfaces in structurally and functionally organised biofilms. Biofilm infections are challenging to treat and antibiotic therapy poses problems of resistance. Since biofilm infections contribute upto 80% of human microbial infections and the oral biofilms associated with caries, periodontitis and diseases like oral cancer. The functional and structural organisation of oral biofilms needs to be studied in much detail.

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