

# Biofilm- The mystery of the oral cavity!

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## Abstract

Microbial biofilms are common in nature. Any fluid environment where bacteria are subjected to stress or flow, can create an environment susceptible for biofilm growth. Biofilms are found throughout the body and in the environment and can be found lining the dental unit waterlines, catheters and prosthetic heart valves. Bacteria exist in the oral cavity in the form of biofilm on tooth surfaces which is a polymicrobial community or consortium of interacting microbial species. It is difficult to grow the microorganisms in artificial culture in the laboratory due to the complex nature of microbial diversity causing periodontal disease. However, due to newer techniques of imaging it has now been possible to understand the biology of dental plaque and knowledge of microbial ecology. Oral biofilms play a major role in the initiation and progression of periodontal disease and have broad implications for quality of life, systemic health and economic costs.

**Keywords:** plaque, biofilm, bacteria, host response.

## INTRODUCTION

Dental plaque has been defined as the microbial community that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Nolte WA et al in 1973 defined dental plaque as nonmineralized microbial accumulation that adheres tenaciously to the tooth surface, restorations and prosthetic appliances, shows structural organization with predominance of filamentous forms, is composed of organic matrix derived from salivary glycoproteins and extracellular microbial products and cannot be removed by rinsing or water spray.<sup>1</sup> Donan and Costerton in 2002 defined biofilm as “a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.”<sup>2,3</sup> Of clinical relevance is the fact that biofilms are less susceptible to antimicrobial agents while microbial

communities can display enhanced pathogenicity.<sup>4</sup> The initiation, formation and adaptation to the fluctuating environmental conditions are ruled by vigorous, continuously changing equilibrium between the microbial species in the oral cavity and a variety of factors that differentially promote or inhibit the survival of its microbial constituents.

### Classification of dental plaque

Dental plaque is broadly classified as supragingival, subgingival or peri-implant plaque based on its location on the tooth and implant surface.

- a. Supragingival
- b. Subgingival
  - i. Tooth associated
  - ii. Tissue associated plaque
- c. Peri-implant plaque

### Composition of dental biofilms

Dental plaque is mainly composed of microorganisms. One gram of plaque contains approximately  $2 \times 10^{11}$  bacteria. 800 distinct microbial species are found in dental plaque. It is composed of 20% solid material and 80% water by composition. The solid material consists of 70-80% microbes and 20-30% intercellular matrix which is the material present in between the bacteria of dental plaque. The microbes include bacterial as well as nonbacterial organisms such as mycoplasma, yeasts, viruses and protozoa. Intercellular matrix is composed of host cells such as epithelial cells, macrophages and leukocytes, organic and inorganic compounds. Organic compounds include polysaccharides such as dextran, proteins such as albumin, glycoproteins that coat the tooth surface and form pellicle and lipid material consisting of debris from membranes of disrupted bacterial and host cells and possibly food debris. Inorganic compounds include calcium, phosphorus, fluoride, potassium and sodium. The source of inorganic compounds for supragingival plaque is saliva whereas for the subgingival plaque, the source is gingival crevicular fluid.

### Stages of plaque formation

Stages involved in the formation of a structurally and functionally organized microbial community include acquired pellicle formation, reversible adhesion involving weak long range physiochemical interactions between the cell surface and the pellicle, coadhesion resulting in attachment of secondary colonizers to already attached cells, multiplication and biofilm formation and detachment.<sup>5</sup> In infants, the predominant microbial species in the oral cavity are positive facultative microbes. Anaerobic bacteria are recovered after the eruption of teeth and the proportion increases in adolescence and adulthood. Various factors affecting the prevalence of microorganisms in the oral cavity include tooth, tooth site, overall health of the dentition, medical status of the patient and their racial origin. Eventually as the oral cavity becomes edentulous with advancing age, there is marked reduction in organisms such as *Aggregatibacter actinomycetemcomitans*, *Lactobacilli*, *S. Mutans*, *S. sanguis* and spirochetes. The organisms that thrive on the epithelial surfaces such as *Candida* continue to thrive.

Minutes after cleaning of the tooth surface, a layer of acquired pellicle is formed, composed primarily of salivary proteins, is formed on the hydroxyapatite crystals. As the formation of acquired pellicle is very rapid after tooth cleaning is highly unlikely that bacterial colonization will take place on any tooth surface without prior formation of acquired pellicle.<sup>6</sup> The first bacteria to colonize the tooth surface are termed as the primary colonizers which are primarily gram positive facultative cocci for e.g. *Streptococcus* species, *Actinomyces*, *Veillonella*. Initial colonization is weak, transient and reversible which eventually becomes stronger. The protein components present in the cell wall of the primary colonizers such as fimbriae, pili and proteoglycans are responsible for attachment of the bacteria to the receptors present on various oral surfaces. These components are known as adhesins.<sup>7</sup> During the first day, the surface is gradually covered by colonies of dividing bacteria that initially spread laterally along the tooth surface. Gradually the proliferating bacteria begin to grow away from the tooth in the form of columnar microbial colonies that are packed closely and compete for space and nutrients with neighbouring colonies. On day 3, filamentous bacteria are found covering the predominantly coccoid plaque. This results in the formation of "corn cob structure" which consists of coccoid bacilli aggregating with filamentous bacteria. The competitive growth for space and nutrients among the coccoid bacilli continues for over 1 week. Then this coccoid population is replaced by predominantly filamentous bacteria covering the surface of acquired pellicle that continues for two more weeks up to several months. Formation of these structures is an indication of plaque maturation as these are stable structures.

Undisturbed plaque formation results in the alteration of the adjacent gingival tissues resulting in typical inflammatory changes including redness and swelling. This results in the pathological deepening of gingival sulcus and formation of a pocket. This pocket provides a suitable environment for the growth of anaerobic bacteria such as motile rods and spirochetes. Rods and filaments tend to be arranged in a palisading pattern with the long axis of the cells perpendicular to the tooth surface. Test tube brush appearance is found in the subgingival plaque in which the bristles of the brush are formed by gram negative

filamentous bacteria that may be flagellated. Majority of these subgingival microbiota are motile due to which a regular stable structure of microbial colonies cannot be seen in subgingival plaque. A layer of neutrophils migrated from the gingival sulcus are present in between the underlying gingival tissues and subgingival plaque. This process of subgingival plaque formation continues up to 3-12 weeks after the beginning of supragingival plaque formation.<sup>8</sup>

#### Relationship of plaque structure to clinical status

The plaque of a periodontally healthy tooth is composed of predominantly gram positive species consisting of facultative aerobic gram positive species. The microbial species associated with gingivitis is characterised by an increase in the microbial mass and in the proportion of gram negative bacteria, motile rods and filaments. Chronic periodontitis microbiota was more complex consisting of predominantly gram negative anaerobic bacteria and spirochetes. The plaque of aggressive

periodontitis affected tooth appears to be less complex and less in thickness as compared to that of chronic periodontitis. The predominant bacteria include *A. actinomycetemcomitans* and fusiform bacilli such as *Capnocytophaga* species.<sup>9</sup>

Routine professional check-up and scaling and root planing prevents the development and maturation of dental plaque by removing it regularly. Thus a hospitable environment is not available for the growth of anaerobic periodontal pathogens. Supragingival plaque control is sufficient for shallow pockets but in pockets deeper than 7 mm supragingival plaque control alone. Waerhaug in 1981 suggested that toothbrushing is able to disrupt subgingival plaque up to 0.9 mm from the gingival margin.<sup>10</sup> This effect may not be sufficient to cause a change in the subgingival plaque composition of deep pockets. This requires a combination of periodontal prophylaxis and home care oral hygiene by the individual with pockets more than 7 mm.

Table 1: Microbiology in periodontal health and disease

Health	Aggressive Periodontitis
<i>Streptococcus sanguis</i>	<i>Actinobacillus actinomycetemcomitans</i>
<i>Streptococcus mitis</i>	<i>Porphyromonas gingivalis</i>
<i>Veillonella parvula</i>	<i>Bacteroides forsythus</i>
<i>Actinomyces naeslundii</i>	<i>Campylobacter rectus</i>
<i>Actinomyces viscosus</i>	<i>Eikenella corrodens</i>
<i>Rothia dentocariosa</i>	
Gingivitis	Refractory periodontitis
<i>Actinomyces</i> species	<i>Actinobacillus actinomycetemcomitans</i>
<i>Streptococcus</i> species	<i>Porphyromonas gingivalis</i>
<i>Veillonella</i> species	<i>Prevotella intermedia</i>
<i>Fusobacterium</i> species	<i>Bacteroides forsythus</i>
<i>Treponema</i> species	<i>Campylobacter rectus</i>
<i>Prevotella intermedia</i>	
Chronic Periodontitis	
<i>Treponema</i> species	<i>Peptostreptococcus micros</i>
<i>Prevotella intermedia</i>	
<i>Porphyromonas gingivalis</i>	
<i>Bacteroides forsythus</i>	
<i>Peptostreptococcus micros</i>	
<i>Campylobacter rectus</i>	
<i>Actinobacillus actinomycetemcomitans</i>	
<i>Eikenella corrodens</i>	
<i>Fusobacterium</i> species	
<i>Selenomonas</i> species	
<i>Eubacterium</i> species	

Plaque as a biofilm- consequence for the organisms

Table a: Microorganisms present in dental plaque in various periodontal diseases and periodontal health

Living in the biofilm community provides many advantages for microorganisms such as:

1. Regulation of gene expression: Bacteria present in the biofilm differ from those present planktonically in the oral cavity (Table a). The attachment of bacteria can result in alteration of gene expression especially in terms of upregulation of exopolymer synthesis. The patterns of gene expression are also changed by the bacteria present in the biofilm. For example, the adhesion of *Pseudomonas Aeruginosa* leads to upregulation of genes involved in alginate synthesis<sup>11</sup> and there is alteration of almost 40-60% of the proteome of this organism.<sup>12</sup> Within the first two hours of attachment of *Streptococcus mutans* to the biofilm, there was a differential expression of 33 proteins and downregulation of 8 proteins. Also, there was an increased synthesis of enzymes involved in carbohydrate metabolism and upregulation of proteins involved in a variety of biochemical functions such as protein folding and secretion, amino acid and fatty acid biosynthesis, cell division and novel proteins of yet unknown functions.<sup>13</sup> These findings suggest that growth in biofilms can have a direct effect (i.e due to initial attachment of bacteria) or indirect (i.e due to altered environmental conditions in the biofilm) effect on gene expression by plaque bacteria.

2. Microbial interactions, cell to cell communication and gene transfer: The microorganisms present in the biofilm interact with each other synergistically or antagonistically. Food chains or food webs are formed as a result of this interaction where the metabolic byproduct of one organism is utilized as nutrient by the other. Synergistically the bacteria collaborate to catabolise host molecules such as proteins and glycoproteins and help each other to survive in an environment conducive for their growth.<sup>14</sup> Antagonistic interactions involve the production of inhibitory compounds such as bacteriocins, acids, H<sub>2</sub>O<sub>2</sub> etc to inhibit the growth of neighbouring cells.<sup>15</sup> Plaque bacteria have a special property of quorum sensing i.e the plaque bacteria can coordinate

their gene expression and communicate with each other in a cell density dependent pattern via small diffusible molecules such as competence stimulating peptide (CSP), lysed cells, acyl homoserine lactone, LuxS genes etc. Certain signalling events can occur between metabolically interacting organisms. For e.g *S.gordonii* secretes more  $\alpha$ -amylase when in co-culture with *Veillonella atypica*. A surface protein of *S.cristatus* can repress *P.gingival* fimbrial gene expression. Communication among the bacteria also occurs by horizontal gene transfer. The signalling molecules such as CSP stimulate the recipient cells to take up DNA by transformation. Tetracycline resistance has been reported by the transfer of conjugative transposons encoding tetracycline resistance. This horizontal gene transfer can result in the evolution of more virulent strains.

3. Tolerance to antimicrobial agents: Bacteria present in biofilm express increased tolerance to antimicrobial agents including those used in dentifrices, mouth rinses and also antibiotics.<sup>16,17</sup> The concentration of chlorhexidine to kill the bacteria present in biofilm is 300 times more as compared to that required to kill planktonic cells. This also depends on the age of the biofilm. Older the biofilm, higher the concentration of antimicrobial required. Studies have indicated that when chlorhexidine is used on 24 hour and 48 hours plaque, chlorhexidine could not penetrate the deeper layers of the biofilm suggesting either quenching of the agent at the biofilm surface or lack of penetration. Bacteria present in the deeper layers of biofilm multiply at a slower pace therefore making them less sensitive to antimicrobial agents.

4. Broader habitat range: The plaque biofilm consists of organisms that are aerobic as well as anaerobic. The primary colonizers are aerobic in nature and consume the oxygen present in the initial stages of biofilm formation thus making the environment anaerobic. This anaerobic environment is conducive for the growth of gram negative organisms which form the majority of the biofilm.<sup>18</sup>

5. More efficient metabolism: Due to the synergistic metabolism between the interacting species present in dental plaque, the host molecules such as glycoproteins are broken down more easily when attacked by a consortium of bacteria.<sup>14</sup> The breakdown

products of these substrates are used as nutrients by some organisms resulting in the development of food chains.

6. Increased tolerance to inhibitory agents and host defences: Neutralizing or drug degrading enzymes are secreted by some organisms in the dental plaque which render the organisms resistant to certain antibiotics. For e.g Gingival cervical fluid (GCF) could have sufficient quantity of  $\beta$ -lactamase to inactivate any penicillin delivered to the site. Also, the periodontal pathogens are protected in a biofilm from the surveillance of host defences such as the action of phagocytic cells.

7. Enhanced virulence: The consortia of bacteria present in a biofilm secrete an array of virulence factors that are responsible for causation of disease by increased pathogenicity and tissue damage. This concept is known as pathogenic synergism.<sup>19</sup>

Methods to determine the oral composition of oral samples<sup>20</sup>

**Culturing-** It is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions. It has an advantage of detecting unrecognized species and provides cultures for further analysis. However it is extremely time consuming, expensive and often difficult to speciate cultures. It is specifically used to study new ecosystems.

**Selective media-** Selective media are used for the growth of only selected microorganisms. They allow certain microorganisms to grow and inhibit the growth of other microorganisms. It is applicable in studies of limited scope involving 1-10 species in modest number of samples.

**Immunofluorescence-** It is a standard technique to identify the presence of antibodies by their specific ability to react with antigens expressed in infected cells; bound antibodies are visualized by incubation with fluorescently labelled antibody. It has the advantage of having specificity and being reasonably rapid although only small number of samples may be run.

**Polymerase Chain Reaction (PCR)-** This method is widely used in molecular biology to make several copies of a specific DNA segment

and identify a particular bacteria. PCR has the advantage of sensitivity and specificity. It is not quantitative of the plaque samples, expensive and dependent on amplification.

**Real time PCR-** This testing combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel. It is quantitative but comparatively slow and very expensive.

**DNA-DNA hybridization-** It is a technique that measured the degree of genetic similarity between the pools of DNA sequences. Its use is confined to the species for which probes are available and requires modest number of species for modest number of samples.

**Checkerboard DNA-DNA hybridization-** It is a method for hybridizing large number of SNA samples against large number of DNA probes. It has the advantage of being sensitive and specific; it can use an entire sample and large number of species. In addition it is inexpensive. It is used in ecology and treatment studies where large numbers of species are studied in large number of samples.

**16S rDNA amplification cloning-** It has the benefit of detecting cultivable as well as uncultivable species and phylogenetic positioning of the taxa. However, it is extremely expensive and extremely small number of samples can be studied with this method.

## Conclusion

Periodontal microbiota is quite complex and differs from site to site and from individual to individual. Periodontitis which results from the dental biofilm is a multifactorial disease, which apart from plaque being the etiological factor, has several other factors influencing the occurrence of the disease such as genetic, immunological, systemic diseases and environmental factors. The diseases which result from the dental biofilm have an overwhelming effect on the oral and systemic health related quality of life. Many advances have been made recently for controlling the formation and management of the consequences of biofilm associated diseases. As a result, there has been a formidable decrease in the prevalence of biofilm associated diseases in the developed countries. Despite the progress, a large portion of the

population is still affected by these diseases. Hence cost effective and sustainable treatment strategies are the need of the hour to further reduce the prevalence of periodontal diseases resulting from dental plaque.

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