‘ORAL BIOFILMS’

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Introduction

Definition

Until recent years, the biological study of microorganisms focused primarily on their colonial life forms (on culture plates) and forms in suspension. New technologies have established that bacteria's fundamental form of life likely involves the formation of aggregates that lead to the creation of communities known as biofilms.

A biofilm is defined as "a sessile microbial community characterised by a first group of cells that irreversibly bind to a substrate or interface, embedded in a matrix of extracellular polymeric substances, which they themselves produce, and they also exhibit an altered phenotype with respect to their growth rate and gene expression". These structures are not passive cell aggregates, but rather dynamic and structurally complex biological systems. This lifestyle allows them to survive in hostile oligotrophic environments, optimising the uptake and distribution of nutrients among the individuals in the community. It also provides them: i) protection against toxins and biocides and ii) enhanced capacity for individuals to perform metabolic and genetic exchange.

In 1978, Costerton et al proposed a theory of biofilm predominance, indicating that it is bacteria's main form of life (Costerton et al., 1978). A clear example of this is found in bacterial biofilm fossils on rocks in hot springs and the ocean floor, which date back 3 billion years. Currently, this form of life thrives in aquatic, natural and industrial environments. In natural environments they are easy to observe in river beds, on rocks and in stagnant water, on rotting tree trunks, etc. From an industrial standpoint, these structures colonise and seriously affect piping and production tanks, even in turbulent flow systems with high shear forces and velocities. They are even able to colonise different types of smooth or rough materials and surfaces.

Such extensive capacity for the colonisation in different environments does not exclude the various surfaces of the human body. And so we find that the skin, the respiratory and digestive mucosa, the oral cavity, etc. are colonised by bacteria in the form of biofilm (Costerton et al., 1999). It is on these surfaces where microorganisms establish commensal and symbiotic relationships with the cells that make up these tissues. These ecological interactions help prevent these areas from being colonised by pathogenic microorganisms. One known example of this is the role that bacteria from the *Lactobacillus* genus play in

vaginal ecology. These bacteria maintain an acidic environmental pH that does not allow colonisation by pathogenic opportunistic bacteria such as *Candida spp*. or *Gardnerella vaginalis*. Generally, an alteration in the delicate balance of bacterial interactions, host characteristics and environmental factors will determine a shift from health to disease.

In the field of medicine, biofilms are a major problem. Statistics show that these life forms are the main source of chronic infection, affecting anatomical structures, including: the oral cavity, the heart, the middle ear, the prostate and pulmonary valves, and cause infectious diseases such as caries, gingivitis, periodontitis, endocarditis , otitis media, chronic bacterial prostatitis, cystitis, cystic fibrosis and sepsis. In 2008, in the United States and the European Union the infectious processes caused by biofilms amounted to 1.7 million infections resulting in 99,000 deaths and 3 million infections with 50,000 deaths, respectively (Bryers, 2008). Biofilms can also settle on different clinical devices or material (prosthetic valves, venous catheters, intrauterine devices, urinary catheters, contact lenses and artificial larynx, etc.).

Oral Biofilm

The oral cavity holds numerous ecological niches in which bacteria develop biofilms (teeth, tongue, mucosa, tonsils, etc.). The composition and distribution of microorganisms in the mouth depend on physico-chemical factors (humidity, salivary pH, temperature and redox potential), adhesion factors to avoid the effects of attrition (intake of food and drink, breathing, saliva flow, oral hygiene, epithelial desquamation), nutritional factors (nutrient uptake through diet and through host secretions) and host protective factors (cell desquamation, saliva, mucosal integrity, the immune system) that push bacterial survival in the mouth to the limit.

It is currently known that the oral cavity is inhabited by more than 700 bacterial species. These species are differentially distributed throughout the various surfaces and, although they settle on mucosal tissues, the biofilms that are most studied are those that develop on teeth, which include supra- and subgingival biofilms (Paster et al., 2001). It is known that 15-20% of the oral biofilm's structural volume is occupied by microorganismos, while the remaining 85% corresponds to their extracellular matrix made up of polysaccharides, glycolipids, proteins and glycoproteins, salts, cell debris, DNA, RNA and water.

Supragingival biofilm is composed mainly of gram-positive aerotolerant bacteria. Some of these species are producers of lactic acid as a result from their saccharolytic metabolism. If the acid concentrations are sufficiently high, a carious process may be triggered, starting with the demineralisation of the inorganic portion of the tooth. Some of these species belong to the *Lactobacillus* genus and others, mainly to the *streptococcos viridans* group. However, when the process reaches the dentin, there appears to be a shift in the microbiota, as the proteolytic activity increases substantially, while the fermentation of sugars is reduced to the minimum (Simón-Soro et al., 2013).

Uncontrolled supragingival biofilm growth provides the basis for the development of subgingival biofilm, which is located in the gingival sulcus, where environmental conditions

and contact with molecules from the immune system determine the type of microbiota that will settle therein. In fact, more than 400 species have been described in this niche, mainly including microaerophilic bacteria and strict anaerobes with a proteolytic metabolism, which in turn have virulence factors determining the pathogenesis of this form of biofilms.

Formation and Development

Biofilm can develop from planktonic cells or from other biofilm. Four hours following a professional cleaning we can find that somewhere between 60 and 90% of the tooth surface has been colonised by species from the *Streptococcus* genus (*S. sanguis*, *S. mitis*, *S. oralis*, etc). These species are an important part of the group of primary colonisers which play a key role in the initial constitution of oral biofilm. These bacteria have a wide battery of adhesins which mediate the intra- and inter-species co-aggregation and the binding of these species to components of the newly formed pellicle (eg . proline rich protein, albumin, glycoproteins, mucins, etc. (Palmer et al. , 2003). Other bacteria that make up this group are *Actinomyces spp, Veillonella spp, Capnocitophaga spp, Haemophilus spp, Propionibacterium spp*., among others. After seven days *in vivo*, the predominant genus continues to be Streptococcus, but other additional species are also observed: *S. gordonii, S. salivarius, S. mutans, S. parasanguis*. After two weeks gram-negative bacteria are observed, which are able to interact with the primary colonisers. The most prominent of these is *Fusobacterium nucleatum*, which has been given a key role in adding more virulent species to the biofilm, known as late colonizers (Donlan & Socransky, 2002).

Socransky and colleagues have suggested that subgingival species are grouped to form microbial complexes, each with a relative importance in the formation and development of biofilms, as well as in their role in periodontal disease. In the gingival sulcus of healthy gums organisms belonging to the yellow and green complexes, mainly primary colonisers such as *Streptococcus spp*, facultative anaerobes and gram-negative bacilli can be detected. Subsequently, bacteria belonging to the orange complex (i.e.: *Prevotella spp* and *Campylobacter spp*.) come into play. Finally, the red complex bacteria, more virulent species and more closely related to the development of periodontitis, *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* are established (Socransky *et al*., 1998).

One of the initial processes, once the cells have been irreversibly bound to the surface, is the formation of the extracellular matrix. For this to occur, cells express the genes necessary for the production of different types of exopolysaccharides (EPS). The matrix also allows for the uptake of cations (Ca^{+2}, Zn^{+2}, Fe^{+3} or Mg^{+2}), sediments and cell debris. Secondary colonisers are attracted by this matrix, thereby diversifying the biofilm. Once the mature biofilm has become established, some bacteria or parts of the biofilm can free themselves and move on to colonise new areas. In general, there are few references regarding the processes, genes, enzymes and structures that are involved in the initiation, maintenance, maturation and release of cells into the environment from biofilms. One of the few examples we have is when *A. actinomycetemcomitans* expresses and releases enzymes, such as dispersin B, which

degrades the exopolysaccharide of the matrix, facilitating the release of cells from a biofilm formed by this species (Kaplan et al., 2004).

The biofilm matrix is a gelatin-like structure whose composition depends on the species contained within. It is made up of water channels by which nutrients and oxygen travel. Its function is mainly structural, as it protects biofilms from physical and chemical aggressions from the environment. Nevertheless, it also provides other advantages, as it can act as a reservoir of nutrients and prevent the penetration of antimicrobials. Recently, Yamanaka et al demonstrated how the production of EPS prevents the phagocytosis of *Prevotella intermedia* through innate cellular response. Because of their inability to phagocytise, neutrophils may end up triggering the release of antibacterial compounds that can damage the surrounding tissue (Yamanaka et al., 2012).

Within a biofilm, there may be a large number of micro-niches or microenvironments. The medium, pH, temperature, flow, dehydration, toxic gradients, the concentration of nutrients, O_2 and CO_2 can stipulate which species can survive at certain depths in the biofilm. This determines why in a biofilm we can find great heterogeneity in the variety and distribution of microorganisms (bacteria [aerobic, microaerophilic and anaerobic], yeasts, fungi and viruses) within a few microns. Aerobic bacteria are in the shallower areas of the biofilm, while less aerotolerant and strict anaerobes are located in the deeper areas.

Clinical Implications

Within these biomasses, microorganisms co-exist, cooperate, interact and communicate with one another. The clinical importance of this intricate ecosystem is that the environment in which it is found is always changing, which breaks the equilibrium that ensures periodontal health. For periodontitis, overgrowth of proteolytic anaerobes, which in conditions of health are detected in very low concentrations both in saliva and subgingivally, can trigger inflammatory reactions and in turn make it more likely for the sulcus to become infected, causing periodontitis. Furthermore, beyond generating infections solely in the periodontium, the consequences of pathogenic biofilm development in the sulcus has been associated with possible impacts on systemic diseases (heart disease, diabetes, osteoporosis, respiratory diseases and pre-term labour).

Infections caused by biofilms are not easily curable and tend to produce recurrent episodes. The intrinsic resistance both to elements of the immune system and to antimicrobials is determined by their structural nature. Furthermore, the bacteria in biofilms have a very low growth rate, which makes the antimicrobial degrade or become inactive before it can exert its effect (Costerton et al., 1999). For these reasons, some authors point out that on the subgingival level, antimicrobials used in periodontal treatment, should be used after mechanically disrupting these biofilms (Herrera et al., 2008). Otherwise, the use of antimicrobials, besides being ineffective, could induce the development of resistance in the bacteria through spontaneous mutation and/or horizontal gene transfer. The use of

antiseptics is also recommended as an aid to mechanical treatment. The mechanism of action of these molecules, especially quaternary ammonium (Cetylpyridinium chloride) and bis-diguanidines (Chlorhexidine) is generic to all microorganisms with negatively charged membranes, hence their spectrum is broader and with less ability to generate resistance.

Study Difficulties and Techniques Used

By controlling these bacterial communities, we increase our knowledge about them. It is necessary to know their diversity, their growth dynamics and their highly varied metabolism. Difficulties that exist for studying oral biofilm are associated with i) the large number of interactions that take place between the different species, ii) technical complexity for analysing internal processes and iii) ethical considerations related to the microbiological study in patients with periodontal disease or caries. These difficulties have led researchers to develop *in vitro* systems for biofilm formation, which aim to simulate the oral environment. These systems use oral, salivary and subgingival plaque inocula or a selection of supra- or subgingival bacteria to develop static biofilms on microtiter plates or in static or flow fermenters (Blanc et al., 2013). Several different microscopic techniques [*Scanning Electron Microscopy* and *Confocal Laser Scanning Microscopy* (CLSM)] and molecular study techniques (*Fluorescence In Situ Hybridization*, *Real Time PCR*, *Microarray* and Massive Sequencing) were used to study the structure, viability and kinetics of biofilm growth from its first stages of formation until maturation.

Oral Biofilms images obtained at Dentaid Research Center

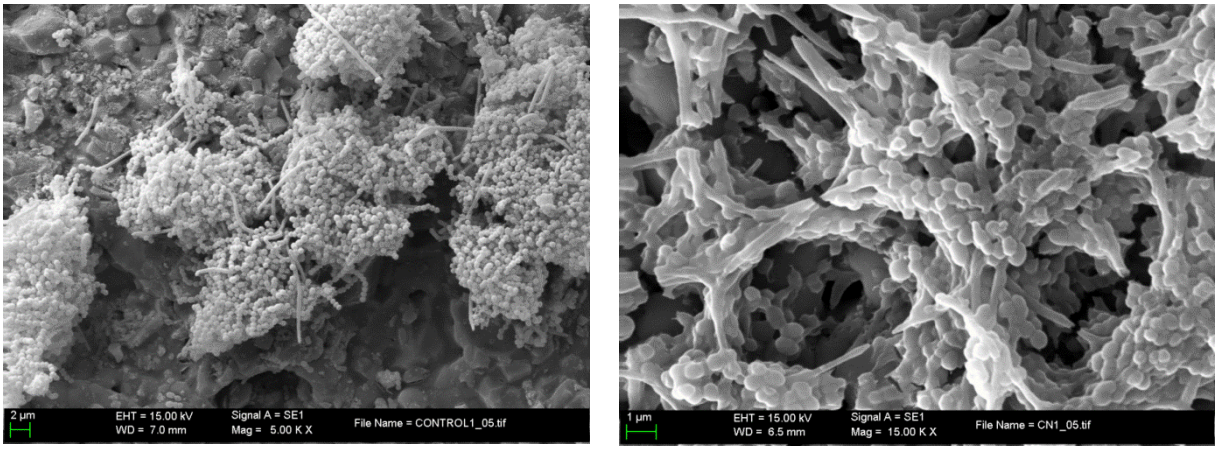

Fig 1. Micrographs of multispecies oral biofilms formed *in vitro* on hydroxyapatite

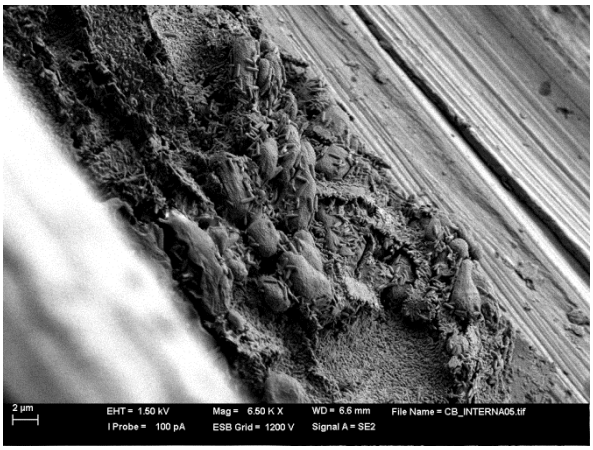

Fig 2. Micrographs of multispecies oral biofilms formed on Ti implants in *in vitro* conditions.

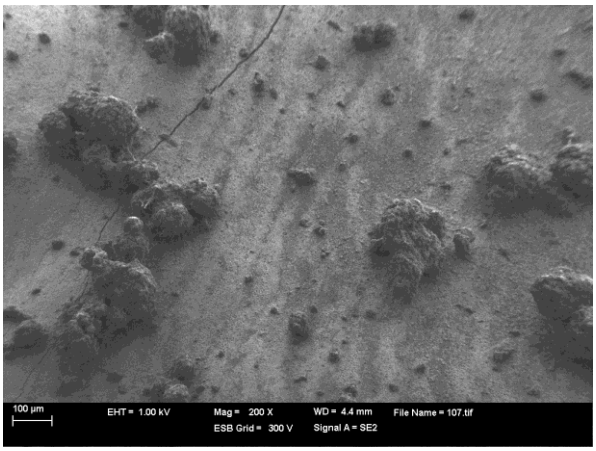

Fig 3. Micrograph of *S. mutans* aggregates on human enamel.

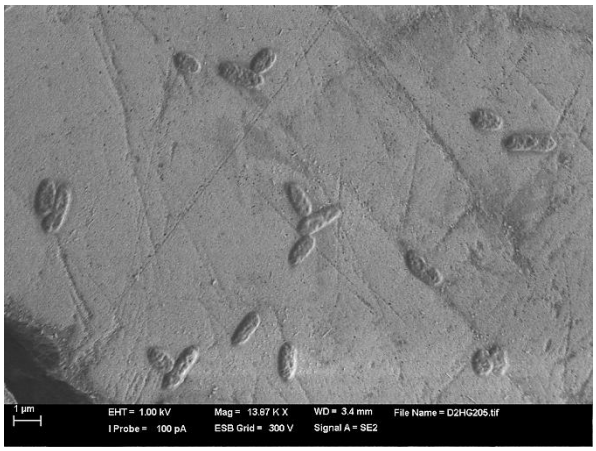

Fig 4. Calcified bacteria on human tooth.

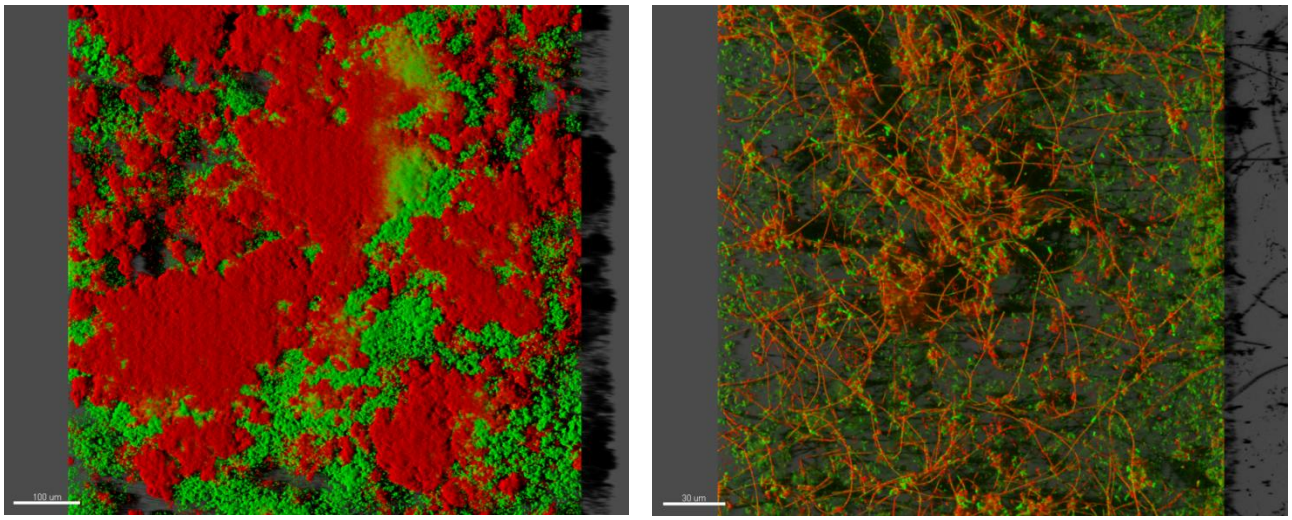

Fig 5. CLSM images of multispecies oral biofilms grown *in vitro*.

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