Chapter

Composition, Structure, and Formation of Biofilms Constituted by Periodontopathogenic Microorganisms

Juliana Cabrini Carmello, Sarah Raquel de Annunzio and Carla Raquel Fontana

Abstract

Microorganisms that compose the oral microbiota maintain complex interactions with each other, especially pathogens related to periodontal disease. It is possible to characterize the etiology of this multifactorial and polymicrobial disease by the accumulation of biofilms formed in the supra- and subgingival environments associated to the immunological response and the susceptibility of the host, being responsible for a large part of the dental loss especially in the adult phase. Periodontal treatment has been carried out mainly by scaling and root planing. This therapy is limited due to the difficult access in some areas of the teeth, impairing the removal of biofilms. So, this chapter will focus on the composition and formation of the biofilm as well as the host's immune response to periodontopathogenic microorganisms. Additionally, the therapeutic challenges and the treatments that are currently being studied in order to eliminate this biofilm, such as antimicrobial phototherapy, will be discussed.

Keywords: bacterial biofilm, periodontal diseases, oral infections, phototherapy, photodynamic therapy

1. Introduction

The human oral microbiota are composed of a wide variety of microorganisms, among the various species of bacteria, fungi, viruses, and protozoa, which live in commensalism, without cause damage to the host [1, 2]. Alterations in the microbial composition due to changes in the environmental conditions or decrease of the host immunity may lead some commensal microorganisms, for instance, *Streptococcus* sp., *Fusobacterium* sp., *Porphyromonas* sp., *Eimeria* sp., *Haemophilus* sp., *Lactobacillus* sp., and *Staphylococcus* sp., to act as opportunists causing infections such as periodontal diseases [2].

Periodontal diseases affect a large part of the population, being one of the main causes of tooth loss in humans [3]. This infection is dependent on the result of the interaction of bacteria with different virulence, present in the dental biofilm, with factors that modify the host immunoinflammatory response [3]. The dental biofilm

is a highly organized structure of microorganisms, in which the microbial species are connected to each other, embedded into an extracellular polymeric matrix forming a highly protective system for the resident species [2, 4, 5].

This infection is not just a local phenomenon, since the microorganisms can penetrate the bloodstream and colonize other niches of the human body, causing bacteremia. Bacteremia is common in individuals who have oral infections, especially in patients with deficient immune systems [2]. Additionally, it has been suggested that there is a relationship between periodontal pathogens and the onset of pulmonary, cardiovascular, diabetes, rheumatoid arthritis, and gestational complications [6–9].

For the treatment of periodontal disease, mechanical removal of the biofilm has been performed as well as the use of antibiotics and antiseptics for bacterial decontamination or as adjuvants to the mechanical removal of the subgingival and supragingival plaque [10]. However, the reinfection occurs very often, and the control of the inflammatory response is difficult. In some individuals, the inflammatory response may reflect a systemic dysregulation, and thus, the resolution of inflammation is impaired using conventional treatment [11]. In this context, phototherapy has been considered as an alternative to antimicrobial agents, such as antibiotics, to suppress subgingival bacterial species and to act as an adjuvant to the conventional treatments to combat periodontal disease.

It is believed that the future in healthcare is to search more efficient treatment alternatives that reduce operating time by improving the final result, eliminating the side effects of the treatment. Thus, the expectancy regarding the application of phototherapy for the treatment of bacterial infections is high, since this therapy has been effective in eliminating the microorganisms present in biofilms without causing systemic side effects to the host tissues.

2. Periodontal disease: classification, epidemiology, and etiology

Recently, a new classification for periodontal diseases has been suggested [12]. In general, the gingivitis can be defined as gingival inflammation caused by bacterial biofilm. Periodontitis includes gingival inflammation accompanied by bone loss and is classified into three different forms: necrotizing periodontitis, periodontitis as a manifestation of systemic disease, and periodontitis. In the last one, periodontitis is classified as "chronic" and "aggressive" [12].

Periodontal disease has been considered multifactorial, episodic, and site-dependent in nature [13–16]. Despite being a multifactorial infection, over the years several studies have demonstrated the importance of microorganisms in the installation and progression of the disease [17–21]. It has been estimated that the presence of plaque and gingivitis is very prevalent in humans, affecting more than 90% of the adult individuals. However, the same cannot be said for periodontitis where, despite the abundance of plaque in most people, the prevalence of periodontitis is relatively low, affecting about 20% of the individuals [22].

In periodontal pockets, the location or distribution of pathogens may be related to periodontal destruction. Noiri et al. [23] reported the presence of *Prevotella nigrescens* in the middle portion of periodontal pockets (epithelial tissue) and the presence of *Fusobacterium nucleatum* and *Treponema denticola* (in areas of non-adherent plaque), related to areas of adhered plaque and *Aggregatibacter actinomycetemcomitans*, in the apical region of the pockets. According to Slots [17] regarding the presence of bacteria in the periodontal pockets, 89.5% were obligatory anaerobic, and 74.9% were Gram-negative. Of all Gram-positive bacilli, 78.4% (deep pockets) and 19.9% (healthy groove) were anaerobic. It can be hypothesized that

gingival inflammation initiated by the supragingival plaque may produce favorable environmental conditions for the colonization of Gram-negative bacteria [17].

In 1988, Socransky and Haffagee [18] reported that destructive periodontal disease depends on the compatible nature of the host or beneficial species colonizing the gingival margin that favors the colonization of other species. Combination of *F. nucleatum*, *Tannerella forsythia* and *Wolinella recta* or *Bacteroides gingivalis*, *Bacteroides intermedius*, and *Staphylococcus intermedius* were associated with sites with greater insertion loss and deep pockets. Clusters of *Veillonella parvula* and *Actinomyces* sp. or combinations of *Streptococcus sanguis* II, *Streptococcus mitis*, *V. parvula*, and *S. intermedius* were associated with sites of lower disease activity and responded more favorably to therapy. Kamma et al. [24] reported that 93.6% of the collected sites presented probing bleeding, and 23.5% were positive for suppuration. *Prevotella intermedia*/*P. nigrescens*, *Porphyromonas gingivalis*, and *Campylobacter rectus* were detected in 77.3–85.9% of the samples using culture methods and in 85.6–91.3% using immunofluorescence. *Peptostreptococcus micros* and *A. actinomycetemcomitans* were found respectively in 63.3 and 25.0% of all sites using culture method and in 58.7 and 27.7% of sites using immunofluorescence. *P. gingivalis*, *T. forsythia*, *P. intermedia*/*P. nigrescens*, and *C. rectus* were observed in 62.1% of the tested sites and 89.4% of the studied patients. The sensitivity found for immunofluorescence of *T. forsythia*, *C. rectus*, *P. intermedia*/*P. nigrescens*, and *P. gingivalis* was high (0.99–0.94) using culture as a reference detection method. The agreement between culture and immunofluorescence in detecting the presence or absence of the investigated species was 85.2–88.1% *for P. gingivalis*, *P. intermedia*/*P. nigrescens*, *C. rectus*, and *T. forsythia*, 75.9% for *A. actinomycetemcomitans*, and 70.4% for *P. micros*.

Comparing the subgingival microbiota of healthy individuals with gingivitis and early periodontitis, using the culture method and DNA probes for hybridization diagnosis, it was initially observed by the culture method that *Bacteroides forsythus*, *Campylobacter rectus*, and *Selenomonas noxia* were predominant species associated with active interproximal lesions. *Actinomyces naeslundii* and *Streptococcus oralis* were dominant in the colonization of active vestibular sites. *Actinomyces naeslundii*, *Campylobacter gracilis,* and *T. forsythia* (at lower levels than periodontitis) were predominant in gingivitis. Health-associated species were *Streptococcus oralis*, *Actinomyces naeslundii*, and *Actinomyces gerencseriae*. By DNA probe diagnosis, higher averages of *Bacteroides forsythus* and *Campylobacter rectus* were identified in periodontitis. *Porphyromonas gingivalis* and *A. actinomycetemcomitans* were detected less frequently in the studied subjects [25].

It has been reported that the microbiota may also vary depending on the teeth involved [26]. Evaluating the microbiota in primary teeth, Kamma et al. [26] found that *Gemella morbillorum* and *Peptostreptococcus magnus* were more frequent in incisive teeth, while *P. micros*, *Streptococcus intermedius*, *Bacteroides forsythus* (*T. forsythia*), *Fusobacterium nucleatum*, *Prevotella loeschei*, *Prevotella melaninogenica*, and *Selenomonas sputigena* were more frequent. The bacterial species *Streptococcus constellatus*, *P. micros*, *Pseudoramibacter alactolyticus*, *Eikenella corrodens*, and *F. nucleatum* were associated with non-blooded sites, while *S. intermedius*, *Campylobacter concisus*, *P. intermedia*, and *Prevotella loescheii* were more frequently found at sites with bleeding [26].

Some authors define that the pathogenesis of periodontitis involves anaerobic bacteria in the oral cavity and that tissue damage occurs as a result of complex bacterial pathogenic interaction and the host's immunoinflammatory response to infection [27–30]. Additionally, although each microorganism has an important role, it is believed that Gram-negative anaerobic rods (*A. actinomycetemcomitans*,

P. gingivalis, *P. intermedia, Bacteroides forsythus, C. rectus, Eubacterium nodatum*, *P. micros*, *S. intermedius*, and *Treponema* sp.), mobile rods, and spirochetes are mainly responsible for causing periodontal disease [31].

As the periodontal diseases are mixed with synergistic infections, it is difficult to determine the role played by a particular species. Studies have shown the relationship of *A. actinomycetemcomitans* with localized aggressive periodontitis and its association with *F. nucleatum*, *P. gingivalis*, *T. forsythia*, and *T. denticola* in chronic periodontitis. *Tannerella forsythia* also shows a remarkable ability to stay in periodontal sites undergoing mechanical or antimicrobial treatment and, because of this feature, is associated with refractory periodontitis [30, 32, 33]. Colombo et al. [34] reported that individuals with refractory periodontitis had a significantly higher frequency of periodontopathogens, such as *Parvimonas micra* (previously *Peptostreptococcus micros* or *Micromonas micros*), *Campylobacter gracilis, Eubacterium nodatum*, *Selenomonas noxia*, *Tannerella forsythia*, *P. gingivalis*, *Prevotella* sp., and *Eikenella corrodens*. In addition to these species, some unusual were also identified: *Pseudoramibacter alactolyticus*, *TM7* sp. *[OT] 346/356*, *Bacteroidetes* sp. *OT 272/274*, *Solobacterium moorei*, *Desulfobulbus* sp. *OT 041*, *Brevundimonas diminuta*, *Sphaerocytophaga* sp. *OT 337*, *Shuttleworthia satelles*, *Filifactor alocis*, *Dialister invisus/pneumosintes*, *Granulicatella adiacens*, *Mogibacterium timidum, Veillonella atypica*, and *Mycoplasma salivarium*. Accordingly, increased proportions of *P. gingivalis*, *Bacteroides forsythus*, *Prevotella, Fusobacterium, Campylobacter*, and *Treponema* species were more prevalent in supra- and subgingival samples from individuals with periodontitis [20].

3. Bacterial plaque: biofilm structure, composition, and formation

The positive association of bacterial plaque (biofilm) accumulation and periodontal tissue inflammation was evidenced in 1965 by Loe et al. [35] establishing the theory of the "nonspecific plaque hypothesis." This theory related gingival inflammation and periodontal destruction from an accumulation of nonspecific microorganisms on the gingival margin. However, later Loe et al. [36] observed that some individuals did not have periodontal disease despite having a large accumulation of gingival plaque, contradicting the "nonspecific plaque hypothesis." Thus, the "hypothesis of specific plaque" emerged, which associates the progression of the disease with the microbial composition. However, this hypothesis did not justify cases in which periodontopathogens were found in places where the disease was not detected or cases in which periodontal disease was diagnosed but microorganisms were not found [37].

In the early 1990s, a new hypothesis called the "ecological plate hypothesis" was described [38]. This hypothesis proposes that the development of gingivitis occurs due to nonspecific plaque accumulation that causes inflammation in the gingival tissues, causing changes in the gingival sulcus environment that make it an environment conducive to the development of Gram-negative bacteria. These environmental changes lead to immunomodulated tissue and inflammatory changes and tissue destruction and result in a greater predominance of periodontopathogens in this microenvironment [22]. This hypothesis corroborates the current concept that the cause of periodontal disease may depend on the host's environmental and immunological factors and not on a particular microorganism or plaque buildup [39]. This concept led researchers to gain a greater understanding of the pathogenesis of periodontal disease [22].

Biofilms that are formed on tooth surfaces and epithelial cells lining the periodontal/gingival sulcus are among the most complex and diverse biofilms formed

by up to 800 different species described so far [40]. It has been reported in the literature that Gram-negative anaerobic bacteria are generally related to periodontal disease. However, facultative anaerobic Gram-positive bacteria are considered beneficial for periodontal health, such as *Streptococcus sanguinis*, which has the ability to produce hydrogen peroxide, which is cytotoxic to *A. actinomycetemcomitans*, a periodontopathogen that is already established in the literature [41, 42].

Bacteria organized in biofilms form microcolonies surrounded by a matrix consisting of extracellular polysaccharides and glycoproteins. This matrix gives protection to bacterial cells and can make these microorganisms up to 1500 times more resistant to antimicrobial treatments in the oral cavity compared to planktonic bacteria [43]. In addition, biofilms are permeated by circulatory channels (which allow the entry and exit of nutrients, metabolites, and residues) and have a mechanism of communication between bacteria called quorum sensing [44]. From this mechanism it is possible to coordinate the bacterial behavior in relation to the environment, being able to regulate the expression of specialized genes according to the population density and to intervene in physiological processes such as the induction of virulence factors [45, 46].

The diversity among the bacterial population in biofilms is due to the existence of microenvironments that present variations in chemical and metabolite concentrations and pH values, so that species with varied metabolic needs can survive [47, 48]. This variety of bacteria present in biofilm ensures that polymicrobial infections caused by dental plaque formed are more difficult to control and makes identifying one or more specific organisms that may be responsible for the infection more difficult [49].

The periodontal biofilm is constantly formed in the supragingival region, and if not removed within 2–4 days, the volume formed will cause this plaque to extend below the gingival margin and into the groove. In a healthy furrow, the number of bacteria found is approximately 10^3; however, in a deep pocket this number can range from 10^8 to 10^{10} [37, 50].

In the process of biofilm formation, subsequent layers of microorganisms bind to existing bacteria through coaggregation. This coaggregation will only occur if these microorganisms share characteristics and/or symbiotic relationships as with the bacteria *T. denticola* and *P. gingivalis*. From the fermentation of amino acids present in the *T. denticola*, gingival plaque produces succinate which is used by *P. gingivalis*, which produces fatty acids which can contribute to *T. denticola* growth [42].

As the bacterial population increases in the biofilm due to the addition of more layers, oxygen runs out making it an environment conducive to anaerobic bacterial colonization [39, 48, 51].

Until the late 1980s, the diagnostic methods used up to now, such as bacterial culture, have not been able to detect and quantify periodontopathogens of subgingival biofilms, given that in this biofilm there are anaerobic bacteria that need adequate growth conditions, besides the difficulty in cultivating the microorganisms that were smaller in the periodontal biofilm samples, preventing the identification and characterization of this biofilm [51]. In this context, in 1998 Dr. Sigmund Socransky described the technique called checkerboard DNA–DNA hybridization for microbiological diagnosis using deoxyribonucleic acid (DNA) probes. From this technique it was possible to develop researches that would improve the knowledge of the periodontal disease microbiota, making it possible to evaluate a large number of samples and microorganisms present in the oral cavity [52].

In this study, Socransky and Haffagee [52] grouped the bacteria in the samples into six complexes named by different colors: red, orange, yellow, green, purple, and blue complex. **Table 1** describes the bacterial species that are part of each

Complex	Bacteria
Red	*Porphyromonas gingivalis*
	Tenarella forsythia
	Treponema denticola
Orange	*Fusobacterium nucleatum*
	Fusobacterium periodonticum
	Prevotella intermedia
	Prevotella nigrescens
	Parvimonas micra
	Campylobacter rectus
	Eubacterium nodatum
	Campylobacter gracilis
	Canpylobacter showae
	Fusobacterium nucleatum ssp. *vicentii*
	Fusobacterium nucleatum ssp. *polimorphum*
	Streptococcus constellatus
Green	*Capnocytophaga sputigena*
	Capnocytophaga gingivalis
	Capnocytophaga ochracea
	Eikenella corrodens
	Aggregatibacter actinomycetemcomitans
Yellow	*Streptococcus gordoni*
	Streptococcus mitis
	Streptococcus sanguinis
	Streptococcus oralis
	Streptococcus intermedius
Purple	*Actinomyces odontolyticus*
	Veillonella parvula
Blue	*Actinomyces gerencseriae*
	Actinomyces naeslundi
	Actinomyces israelli

Table 1.
Representation of bacteria divided into complexes established in the study by Socransky and Haffagee [52].

complex. Bacteria that were grouped in the red complex are considered as etiological agents of chronic periodontitis and related to gingival bleeding and increased pocket depth. The bacteria under the complex named orange, which proceeds the installation of the red complex and its constituents, are considered possible periodontal pathogens. The complexes named green, yellow, purple, and blue are integrated by bacteria that colonize the dental surface in the early stages of biofilm formation and are compatible with periodontal health. However, these complexes provide receptors and provide an ecosystem conducive to the emergence of bacteria present in the orange complex and in turn the red complex, which are in fact related to the pathogenesis of periodontal disease.

4. Host immune response to pathogenic microorganisms

The main periodontopathogens, such as *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, and *T. forsythia*, have important proteolytic and exopeptidase activity, which have trypsin-like activity. In *T. denticola* these proteases behave like chymotrypsin-like serine proteases and are responsible for the invasion of this microorganism into tissues. Moreover, they play an important role in the development of necrosis in periodontal disease and amino acid fermentation by releasing ammonia, hydrogen sulfide, methyl mercaptans, and highly toxic fatty acids, which exert direct cytotoxic activity and reduce the speed of tissue repair [53–55]. *A. actinomycetemcomitans* is capable of producing an active thermolabile leukotoxin on neutrophils, monocytes, macrophages, and T lymphocytes, producing degranulation of these cells, with subsequent tissue disorganization and local immunosuppression [56, 57].

A specific bacterial etiology for the development of periodontitis from longitudinal studies with individuals infected with *A. actinomycetemcomitans* has been suggested [58]. A cohort study of 96 students included a test group of 38 students positive for *A. actinomycetemcomitans* and 58 healthy controls for this bacterium. The patients were studied longitudinally for 2–3 years. During the study period, 7 of the 37 individuals that are actinomycete-positive (i.e., 18%) developed bone loss compared to none of the *A. actinomycetemcomitans*-negative subjects. The authors suggested that *A. actinomycetemcomitans* is a significant risk marker for the development of aggressive periodontitis [58].

The interaction between the host and the microorganisms is clearly responsible for the development of gingivitis injury. With regard to periodontitis, it can be argued that the specific bacteria observed so far are present as a result of the disease, but not necessarily caused the disease. This argument is no different from the most mucosal bacterial biofilm infections in which the relationship between disease and inflammation is not clear. What comes first: host response or change in biofilm microorganisms? [59].

Although many studies evaluate the subgingival microbiota of healthy and diseased periodontal sites, further investigations are needed to fully understand these infections and host-pathogen interaction and to study new treatment options for this disease. One such approach is the phototherapy or photodynamic therapy described below.

5. Conventional treatments and therapeutic challenges

The treatment of periodontal disease is focused on the elimination of biofilm and calculus and the prevention of its formation. As a conventional treatment, scaling and root planing (SRP) is performed by removing plaque accumulation and calculating below the gingival margin, preventing disease progression and bacterial recolonization on the tooth surface [60].

This treatment has caused a decrease in pathogens, considering that after this procedure, it was reported in the literature that the bacterial load of *T. denticola* and *P. gingivalis* was reduced after 1 year of SRP. In addition, this treatment has other benefits, such as the gain in clinical insertion level and reduction of periodontal pocket depth [61, 62].

However, this procedure is limited due to the technical difficulty in removing biofilms located in hard to reach areas, such as very deep periodontal pockets, root concavities, bifurcations, and large invaginations. Additionally, a possible relapse may occur as some periodontopathogens such as *A. actinomycetemcomitans* and

P. gingivalis can invade the tissue, so the persistence of these bacteria on the root surface can cause recolonization in sites that have already been treated [39].

In order to optimize the effects of SRP treatment, protocols have been proposed to associate systemic or local antibiotics to eliminate persistent bacteria after the SRP procedure. Studies have shown that this association provides improvement in the patient's clinical condition [63]. The main antibiotics commonly used in the treatment of periodontal disease are amoxicillin, metronidazole, clindamycin, azithromycin, ciprofloxacin, doxycycline, and minocycline [64]. However, the use of these drugs as an adjuvant to this disease has limitations, such as the emergence of bacteria resistant to these antibiotics as well as the side effects caused by these antimicrobial agents, such as diarrhea and vaginal candidiasis, which result from the commensal microbiota imbalance. In addition, drug interaction may occur between antibiotics and other drugs being used by patients, resulting in ineffectiveness or other adverse effects [49, 65].

Thus, in recent years, in the area of dentistry, promising antimicrobial adjuvant therapies have been studied, such as phototherapy and photodynamic therapy [66, 67].

5.1 Phototherapy and photodynamic therapy

Studies have shown that some bacteria related to periodontal disease have the ability to produce a photosensitive substance intrinsically, such as protoporphyrin IX. Even without the addition of a photosensitizing drug, pigmented bacteria have been more susceptible when applied to phototherapy [66, 68]. Photosensitizers are molecules that when irradiated by a light source at a suitable wavelength undergo photochemical reactions to emit fluorescence. This process is used by photodynamic therapy to produce reactive oxygen species [69, 70]. Most bacteria do not have endogenous photosensitive compounds. Thus, cells lacking these compounds may become susceptible to light when an exogenous photosensitizing molecule is added [71, 72].

The mechanism of action of photodynamic therapy happens when the photosensitive substance (intrinsic or extrinsic) is activated when irradiation is applied by a light source compatible with the length of the substance. This process will form reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, causing the death of the bacteria. This interaction of light and photosensitizer can occur through two types of reactions, called type I and II. In the type I reaction, charge transfer occurs between the photosensitizer and biomolecules, resulting in radicals and radical ions that react with molecular oxygen, forming reactive oxygen species. During the type II reaction, the excited triplet state photosensitizer transfers energy directly to the fundamental triplet state oxygen, forming singlet oxygen [69, 73, 74].

Studies involving photodynamic therapy and periodontal disease have investigated different light sources such as light-emitting diodes, low-power lasers, and conventional light [75–79]. As for photosensitizers, there are several molecules studied aiming at inactivation of periodontopathogens such as poly-L-lysine-chlorin-6 conjugate and phenothiazine dyes (toluidine blue and methylene blue) [80, 81].

Photodynamic action is being increasingly studied to complement the microbial reduction achieved by conventional mechanical periodontal therapy. In vitro studies have shown that periodontopathogens have been suppressed in planktonic phase and biofilm, and after the application of photodynamic therapy, it has been verified that virulence factors of these bacteria have been decreased, such as lipopolysaccharides and proteases [82–84].Clinical trials have also shown that this therapy is effective as an adjuvant in the treatment of periodontal disease [85–87]. However,

several parameters must be considered for this therapy to be successful, such as the photosensitizer used, its concentration, and the irradiation parameters. Thus, further studies should be conducted to develop clinically applied protocols.

6. Conclusions

The etiology of periodontal disease is multifactorial and directly associated with biofilm accumulation in the supra- and subgingival region, immune response and host susceptibility. In recent decades, several studies have sought to investigate the complex interactions of periodontopathogens in biofilm as well as adjuvant antimicrobial therapies that do not cause adverse effects in patients nor bacterial resistance. Phototherapy and photodynamic therapy are examples of treatments that have shown promising results in vitro and in clinical trials. Further investigations need to be done in order to establish parameters which allow the safe and efficient application of the therapy.

Conflict of interest

The authors declare no conflict of interest.

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