Interactions among cariogenic bacterial species in oral biofilm

Rosa Virginia Dutra de Oliveira[1], **Cristiane Yumi Koga-Ito**[2] and **Fernanda Lourençao Brighenti**[1]

[1] UNESP, Department of Orthodontics and Pediatric Dentistry, Araraquara School of Dentistry, Univ Estadual Paulista/UNESP, Rua Humaitá, 1680, Centro, 14801-903, Araraquara, SP, Brazil
[2] UNESP, Institute of Science and Technology, São José dos Campos, Univ Estadual Paulista/UNESP, Av. Eng. Francisco José Longo, 777 - Jardim São Dimas, 12245-000, São José dos Campos, SP, Brazil

Biofilm is considered a complex microbial community, in which microorganisms with different growth requirements can co-exist. During biofilm maturation, bacteria tend to develop strategies in order to facilitate their establishment and survival. These strategies include: coadhesion; coaggregation; competition for nutrients; production of metabolic products; modulation of virulence factors; quorum sensing; genetic material exchange; and resistance to antimicrobials. Within oral biofilm, the proximity of microorganisms facilitates synergistic and antagonistic interactions between neighbouring species. This chapter will discuss the role of these interactions in balancing competition/coexistence and how relationships among microorganisms may influence the occurrence of dental caries.

Keywords: Microbial interactions; Biofilm; Microbial Antagonism; Microbial Cooperative Behaviour

1. Introduction

The oral biofilm formation is a coordinated process, involving distinct stages, which includes initial attachment of primary colonizers to acquired pellicle, adhesion of secondary colonizers, multiplication, maturation and detachment [1,2]. The acquired pellicle is a thin protein containing film derived from salivary glycoproteins, which serve as a source of receptors for primary colonizers [3]. Then, secondary colonizers adhere via cell-surface adhesins to receptors on previously attached bacteria [4]. (Kolenbrander et al., 2002) If environmental conditions are favorable, cells start to multiply and the substratum becomes covered by bacteria, thus the biofilm begins to develop into a multispecies community [5]. The high cell density and the close cell-to-cell contact results in intra- and interspecies interactions that can benefit or antagonize the involved microorganisms, as well as influence the community composition [6,7].

In order to facilitate their establishment and survival within the biofilm, bacteria tend to develop strategies. These strategies include: coadhesion, coaggregation, competition for nutrients, production of metabolic products, modulation of virulence factors, quorum sensing, genetic material exchange, and resistance to antimicrobials [8].

The present chapter focuses on the interactions among bacterial species and how these interactions contribute to the development of dental biofilm, as well as provides an overview of the physical and metabolic interactions that occur among the oral microflora in the context of cariogenic biofilm development.

2. Interactions between microbial species in dental biofilm

2.1 Coadhesion and coaggregation

Streptococci have identified as the predominant colonizers of oral biofilm, which compose about 63% of bacteria isolated in early enamel biofilm [9-13]. Most of streptococci are able to directly bind to receptors in the salivary pellicle [14]. However, sometimes different species compete for binding to these salivary receptors. *Streptococcus gordonii* is known as a strong competitor of *Streptococcus sanguinis* in adhesion to saliva-coated hydroxyapatite [15].

Bacteria may also bind indirectly to the acquired pellicle using receptors of partners [16]. The adherence of a bacterium to a previously attached bacterial cell is called coadhesion. Another common process is coaggregation, which is the adhesion among bacteria in suspension [17]. Both processes may occur between genetically distinct bacterial partners and usually they are specific, since it is mediated by adhesin–receptor pairs [14].

Some bacterial species may recognize the same receptor on a partner, competing for binding sites [7]. An example of coaggregation competition occurs between *Actinomyces* and *Prevotella*, which recognize the same receptor on streptococci.

Coaggregation and coadhesion enable physical interactions among bacterial species and attachment to surfaces, thus, contribute to changes on the biodiversity in biofilm and play a key role on bacterial succession [18,7].

2.2 Competition for nutrients

The association of an organism with a particular habitat is directly related to nutritional requirements [6]. The availability of nutrients may influence the bacterial composition of the biofilm [8]. Mutans and mitis groups streptococci are found in the same niche of the supragingival biofilm and have similar nutritional requirements. Epidemiological studies have found an inverse relationship between members of these two groups: high numbers of

Streptococcus sanguinis, a member of mitis group streptococci, were correlated with low numbers and delay in the colonization by mutans streptococci [9,19]. Although it is not the only factor, the nutritional availability plays important role in determining the outcome of this competition.

Competition assays *in vitro* and in biofilms demonstrated a mutual exclusion between *Streptococcus mutans and S. sanguinis* depending on the sequence of inoculation and revealed that cell density, production of inhibitory substances and pH can also modulate competition and coexistence of these two species [20].

In general, *S. mutans* is also known as a fierce competitor of the oral biofilm because of its capacity to quickly metabolize different carbohydrates, producing elevated amounts of extracellular polysaccharides. These polysaccharides provide support to development and accumulation of microcolonies and increase the cohesiveness and structural integrity of the biofilm [21]. *S. mutans* is related to higher caries incidence [12, 22-24] and *S. sanguinis* is able to antagonize it. These findings suggest that *S. sanguinis* may be associated with health [25] and provide new insights into ecological approaches toward controlling dental caries [9].

The type of the nutrient may also influence the competitiveness. In the presence of glucose, the ability of *S. sanguinis* to inhibit growth of *S. mutans* was slightly reduced, because of the repression of hydrogen peroxide (H_2O_2) production [11]. Hydrogen peroxide is considered an inhibitory substance produced by some streptococci which would contribute to the antagonism between *S. mutans* and *S. sanguinis.*

2.3 Production of metabolic products

Metabolic products by one organism may affect others within the biofilm. Previous studies have shown that bacteriocins, peptides produced by oral streptococci, are able to lyse other bacteria [26] or act as analogues of signalling molecules [8].

S. mutans bacteriocins are termed mutacins. It has been suggested that these mutacins may be related to the successful of *S. mutans* establishment in the biofilm [11,20,26] and also to higher prevalence in the oral cavity of subjects with high caries experience [Giacaman et al., 2015]. Mutacins I and IV are able to inhibit *S. sanguinis* [20]. Kreth and cols. determined the prevalence of mutacin I and IV gene in *S. mutans* clinical isolates [20]. Mutacin IV gene was detected in at about 50% of the samples, whereas both mutacins I and IV genes were detected in at about 5% [27].

The large amount of organic acid produced by *S. mutans* also acts as a mechanism of inhibition. In the presence of high levels of glucose, *S. mutans* is able to produce significant amount of lactic acid, due to the higher activity of ATP-glucose phosphotransferase and repress the growth of *S. sanguinis* [28]. It was also observed that glucose may repress the expression of pyruvate oxidase, which is related to hydrogen peroxide (H_2O_2) formation. Thus, glucose may indirectly inhibit the excretion of H_2O_2 [16].

Hydrogen peroxide is a metabolic product excreted by some oral streptococci, such as *S. sanguinis* and *S. gordonii,* responsible to inhibit peroxide-sensitive species [11]. *S. mutans* growth inhibition was observed in the presence of hydrogen peroxide, since this species does not express effective systems for metabolizing this toxic product [20]. In addition, H_2O_2 may repress *S. mutans* genes virulence [19].

The competitiveness between *S. sanguinis* and *S. gordonii* over *S. mutans* increased in presence of oxygen, which is used to produce H_2O_2 [11]. These authors observed that when *S. sanguinis* and *S. gordonii* were inoculated first, under aerobic conditions, the inhibition of *S. mutans* was increased. On the other hand, in anaerobic environment, no inhibition over *S. mutans* was observed.

An epidemiological study also seems to support the idea of antagonism between *S. sanguinis* and *S. mutans* within the dental biofilm. Giacaman and cols. observed that higher numbers of *S. sanguinis* were isolated from the saliva of adults without caries experience, whereas *S. mutans* predominated in high caries prevalence adults [19]. Additionally, *S. sanguinis* colonies isolated from caries-free subjects produced more H_2O_2 *ex vivo* than those with high caries experience.

Another fierce antagonist of *S. mutans* is *Streptococcus oligofermentans*. Because this species metabolizes fewer carbohydrates, produces less acid and seems to only exist in healthy people, it has been suggested that *S. oligofermentans* probably is a non-cariogenic species [29]. Surprisingly, *S. oligofermentans* is able to metabolize the lactic acid produced by *S. mutans* and convert into hydrogen peroxide by using lactate oxidase activity [30] or pyruvate oxidase [31]. In addition, this species produces H_2O_2 from L-amino acids [32]. This inhibitory effect seems to be relatively specific to *S. mutans* and it was attributed to production H_2O_2 [30,33]. However, *in vitro* studies observed that the presence of oxygen and carbohydrates, pH and the sequence of inoculation may also affect the inhibition effect [33,34]. The inhibitory effect was enhanced when the bacteria were cultured with carbohydrates and under aerobic conditions [33]. Besides, pH 7.0 was the optimal pH for *S. oligofermentans* growth and the most pronounced inhibitory effect was observed when it was inoculated first [34].

In addition to antagonistic effects, certain bacterial species can modify the local microenvironment by production of substances, which make it more suitable for the growth of other species [8]. The lactic acid produced by *S. mutans,* which inhibit *S. sanguinis*, may benefit *Veillonella parvulla* growth in dual-species biofilms [35]. The opposite situation is also observed: when cultured with *V. parvula*, *S. mutans* grew well or better than in single-species biofilms and exhibited few alterations on genes expression [13]. Additionally, the survival rate of *S. mutans* and *V. parvula* in dual-species biofilms after chlorhexidine treatment was higher than in single-species [36].

Actinomyces naeslundii is a pioneer species, which is able to synthesize catalase, removing H_2O_2 from coaggregate cultures, protecting peroxide-sensitive species [2]. As mentioned above, *S. gordonii* produces hydrogen peroxide at concentrations sufficient to kill other species, but at the same time, accumulation of this metabolic product could induce deleterious effect on itself [37]. Coaggregation with *A. naeslundii* enhances growth and survival, as well as protects *S. gordonii* against oxidative stress [38].

Although lactobacilli have been considered as cariogenic microorganisms for a long time [39], some species are known to play a role in the maintenance of human health by stimulating a native immunity and protection against infection [40]. Because of these benefits, these species have been termed probiotics and tested as a preventive strategy to control oral biofilm formation [41]. In 2007, Simark-Mattsson and cols. investigated the inhibition capacity of lactobacilli isolated from subjects with and without caries against mutans streptococci [42]. Lactobacilli isolated from subjects without caries experience, inhibited the growth of mutans streptococci more effectively. These subjects also exhibited lower colonization by *S. mutans* [42]. Strong inhibitory activities were associated with *Lactobacillus paracasei, L. plantarum, L. rhamnosus, L. casei* and *L. salivarius* [42, 43]. It has been suggested that lactobacilli probably produce bacteriocins [44, 45]. Recently, it was reported that *L. reuteri* was also able to completely inhibit the growth of *S. mutans* [46]. The antibacterial activities of *L. reuteri* were attributed to the production of organic acids, hydrogen peroxide and a bacteriocin-like compound.

2.4 Modulation of virulence factors

Bacterial interactions can affect the growth of other species, which could have specific effects in terms of the virulence properties and influence the pathogenicity of biofilm [8]. In this regard, organisms able to control the amount of the acidic end products would contribute to reduce biofilm acidogenicity and thus, the development of dental caries. Wu and cols. analysed different *Lactobacillus salivarius* strains and found that two (K35 and K43) showed more pronounced inhibitory activities against *S. mutans* biofilm formation. It was observed that the expression of *S. mutans* virulence genes which encode glucosyltransferases gtfB, gtfC, and gtfD was reduced, nevertheless this is not a general characteristic of the species [47].

Also, *V. parvula* readily metabolizes lactic acid produced by *S. mutans* into weak acids such as propionic and acetic acid, which may lead to a less cariogenic environment [35]. Furthermore, *Veillonella* species may utilize lactate as energy source for growth [48, 49]. Nevertheless, despite the conversion of lactic acid into less potent acids, Becker and cols. observed that *Veillonella* species were found in association with streptococcii in caries lesions [50]. Both species are highly correlated with total acid producing [51]. Thus, more studies are necessary to better understand these interactions.

Even more interestingly is *Actinomyces naeslundii,* which depending on the presence or absence of oxygen is able to reduce or increase the cariogenicity of the biofilm. Under aerobic conditions, *A. naeslundii* can metabolize carbohydrates into relatively weak acids, stabilizing the pH of the environment [52]. On the other hand, under anaerobic conditions this bacterium produce more acids, whose accumulation promote acidification of the environment and consequently colonization of more acidogenic and acid-tolerant bacteria [52].

2.5 Quorum sensing

Quorum sensing (QS) is the self-induced secretion of signalling molecules called autoinducers, in response to changes in bacterial density at the surrounding environment [16]. Quorum sensing (QS) plays an important role in biofilms by controlling functions, such as bacterial surface adhesion and extracellular matrix production, [53] biofilm maturation [54], release of extracellular DNA [55, 56] and antimicrobial production [57].

When in co-culture with *Streptococcus gordonii, Veillonella atypica* is able to upregulate *S. gordonii* amylase gene expression, increasing amylase activity [48]. It was suggested that the interspecies communication was mediated by diffusible signalling molecules based on quorum-sensing system. The *S. mutans* quorum-sensing system is composed by competence-stimulating peptide (CSP) [58] The production of CSP may be induced under stress conditions and, when in high concentrations, could lead to autolysis in a fraction of *S. mutans* population [59].

2.6 Genetic material exchange

The close proximity of the residents within biofilms enable gene transfer between the species [2]. The release of extracellular DNA (eDNA) may be induced by bacteriocins, since they can cause cellular lysis [60]. It was observed that the release of eDNA by *Streptococcus sanguinis* and *Streptococcus gordonii*, occurs in response to hydrogen peroxide production [61]. Interestingly, this process does not cause cellular lysis, but eDNA contribute to genetic material exchange, as well as adhesion of these bacteria to dental surface [61]. According to Itzek and cols., the production of hydrogen peroxide could serve indirectly as trigger for antibiotic resistance genes transference, besides mutations, since DNA repair mechanisms do not work extracellularly [62].

Extracellular DNA also enhances *S. mutans* adhesion, probably due to interaction with glucans [63]. It was suggested that eDNA may facilitate cell-cell adhesion [64], play role as a matrix component [65], act as a nutrient store [66], stabilize the structural integrity of the biofilm [14] and allow the exchange of antibiotic resistance markers [8,60].

Some microorganims are naturally able to obtain eDNA [14], however others need a small molecule termed competence-stimulating peptide to become competent [67]. Kreth and cols. found that *S. mutans* needs to produce and to release mutacin IV in order to induce eDNA release to the neighbouring species. In response to secreted CSP, *S. mutans* become competent to acquire eDNA [20]. It was also suggested that *S. mutans* CSP acts as a quorum-sensing regulator [68].

2.7 Resistance to antimicrobials

In vitro studies confirmed that microorganisms within the structure of biofilms are more resistant to antimicrobials than planktonic cultures [69-72].

Bacterial cells grown in biofilm tend to express different properties [70], phenotypes [69] and specific spatial arrangement, which may contribute to the survivability and resistance to antimicrobials [73]. It is believed that the spatial arrangement in clusters is related to mutualistic interactions between the species and may have been responsible for the higher rate of survival after exposure to chlorhexidine in dual-species biofilm of *S. mutans* and *V. parvula* [36]. Corbin and cols. also found that clusters in the central area of the biofilm were less susceptible to antimicrobials than cells near the cluster edge [74].

Few years ago, an *in situ* study suggested that the remaining biofilm could contribute to extend the substantivity of oral antimicrobials [75]. However, another in situ study observed a progressive recovery in bacterial vitality after the use of 0.2% chlorhexidine mouthrinses [76]. According to He and cols., the remaining biofilm tends to have lower water content, which means that to penetrate deeper within the biofilm, antimicrobials would be more diluted and, consequently, less effective [77]. Another possible explanation for this phenomenon would be the presence of exopolysaccharide matrix, which could protect the microorganisms from the direct action of antimicrobials [78] and thus, contribute to the survival of bacterial cells [35]. The polymeric matrix may provide mechanical stability to the biofilms and act as a barrier [79], affecting the diffusion of substances through the biofilm [74, 80]. Furthermore, findings suggest that the habitual application time of antimicrobials it is not enough to eradicate bacteria within the biofilm [81]. Remaining bacteria and constituents derived from disrupted cells may persist and prevent the diffusion of antimicrobials, as well as protect those that are at deeper sites [82].

As mentioned above, another way to acquire antimicrobial resistance is by means of genes transference. The proximity among bacterial cells within the biofilm can facilitate the material genetic exchange and thus, the transference of antibiotic resistance genes [8, 14]. *Acinetobacter baumannii,* an inhabitant of oral biofilm associated with periodontitis [83], is able to transfer antibiotic resistance genes by conventional horizontal gene transfer and using vesicles [84]. It is believed that the horizontal gene transfer is the main mechanism responsible for spread of antibiotic resistance genes [85] and the oral microflora could serve as a reservoir for antibiotic resistance determinants [86]. Loyola-Rodriguez and cols. observed that the most resistant species found in primary dental infections were: *Streptococcus oralis* and *Prevotella intermedia* (75.0%); *Treponema denticola* and *Porphyromonas gingivalis* (48.3%); *Streptococcus mutans* (45.0%); *Campylobacter rectus* and *Streptococcus salivarius* (40%) [87].

The release of extracellular DNA within the oral biofilm could also considered a way to donate and acquire antimicrobial resistance genes [62].

3. Conclusion

It is known that interspecies interactions can influence the composition of the oral biofilm. The success on the establishment of which each species is related to the ability to use available resources and tolerate adverse conditions.

An interesting approach to inhibit biofilm virulence may be the prevention of pathogenic organisms incorporation. Also, to enhance the colonization and growth of organisms able to antagonize potentially cariogenic species. Thus, the understanding of these interactions could indicate new possibilities and strategies for prevention of dental caries.

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