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ROSA VIRGINIA DUTRA DE OLIVEIRA

INTERAÇÃO ENTRE BACTÉRIAS CARIOGÊNICAS

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ROSA VIRGINIA DUTRA DE OLIVEIRA

INTERAÇÃO ENTRE BACTÉRIAS CARIOGÊNICAS

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ROSA VIRGINIA DUTRA DE OLIVEIRA

NASCIMENTO	29 de setembro de 1981 – João Pessoa/PB
FILIAÇÃO	Jair Santiago de Oliveira Alzir Rosa Dutra de Oliveira
2000 - 2004	Graduação em Odontologia Universidade Federal da Paraíba – UFPB
2004 - 2005	Aperfeiçoamento em Endodontia Núcleo de Estudos e Aperfeiçoamento Odontológicos - NEAO
2005 - 2006	Aperfeiçoamento em Periodontia Núcleo de Estudos e Aperfeiçoamentos Odontológicos- NEAO
2005 - 2007	Especialização em Endodontia Clínica Integrada de Odontologia – CIODONTO
2007 - 2008	Especialização em Programa de Saúde da Família Faculdade de Ciências Médicas – FCM

2008 - 2009	Especialização em Saúde Pública Faculdade de Ciências Médicas – FCM
2010 - 2011	Mestrado em Odontologia Preventiva Infantil Universidade Federal da Paraíba – UFPB
2012 - 2016	Doutorado em Odontopediatria Faculdade de Odontologia de Araraquara – UNESP
2014 - 2015	Estágio Sanduíche Center for Biofilm Engineering – Montana State University

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RESUMO

A proximidade entre micro-organismos presentes no biofilme dentário determina a existência de interações entre eles, que podem beneficiar ou antagonizar as espécies envolvidas. Os objetivos deste estudo foram: 1) apresentar uma revisão de literatura sobre interações entre espécies bacterianas cariogênicas no biofilme oral; 2) validar o uso do reator ‘‘drip-flow’’ (DFR) para desenvolver biofilmes dentários e testar agentes antimicrobianos; 3) avaliar o crescimento e a suscetibilidade à clorexidina de biofilmes compostos por *Streptococccus mutans* ATCC 25175 e *Lactobacillus acidophilus* ATCC 4356, usando o DFR; 4) avaliar o crescimento e a suscetibilidade à clorexidina de biofilmes compostos por *Streptococccus mutans* e *Actinomyces naeslundii* ATCC 12104, usando o DFR. Biofilmes cresceram sobre lâminas de vidro cobertas com hidroxiapatita e caldo BHI suplementado com sacarose 0.2% ou 0.5%, dependendo das espécies e um fluxo de 10 mL/h foi usado. O DFR foi incubado por 24 h a 37 °C / 5% CO_2. Biofilmes foram tratados com clorexidina 0.2% (CHX) ou NaCl 0.9% por 2 min. Crescimento e efeito dos tratamentos foram determinados por contagem de unidades formadoras de colônias (UFC). Biofilmes foram marcados com o kit de Viabilidade *Live/Dead* e analisados por microscopia confocal de varredura a laser para diferenciar células com membranas íntegras das lesadas pela ação da CHX. A validação do DFR foi analisada por teste t (α=0.05). Avaliação da interação foi analisada por ANOVA dois fatores e teste de Tukey (α=0.05). A revisão de literatura mostrou o papel das interações microbianas no balanço competição/ coexistência. Na validação do DFR, resultados mostraram que, apesar da diferença inicial das concentrações de mono-culturas de *S. mutans*, o tratamento com clorexidina afetou ambos os biofilmes na mesma proporção. Não foi observada interação entre solução de tratamento e condição de cultura em biofilmes de *S. mutans* e *L. acidophilus*. Entretanto, a viabilidade foi significativamente reduzida após o tratamento com CHX. Mono-culturas de *L. acidophilus* cresceram significativamente menos que ambas mono- culturas e culturas mistas de *S. mutans*. *S. mutans* e *A. naeslundii* cresceram similarmente em ambas condições de cultura, dentro do grupo NaCl 0.9%. A viabilidade bacteriana foi significativamente reduzida em todos os grupos tratados com clorexidina, exceto para culturas mistas de *S. mutans*. Mono-culturas de *A. naeslundii* foram as mais suscetíveis, enquanto culturas mistas de *S. mutans* forma as menos suscetíveis à CHX. Em conclusão, relações entre os micro-organismos podem influenciar a ocorrência de cárie dentária. O presente estudo mostrou a aplicabilidade do DFR para crescer biofilmes orais e testar o uso de agentes antimicrobianos. Foram encontradas interações significantes entre *S. mutans* e *A. naeslundii*, mas não entre *S. mutans* e *L. acidophilus*.

Palavras-chave: *Streptococcus mutans*. *Lactobacillus acidophilus*. Actinomyces. Clorexidina. Cárie dentária. Biofilmes.

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ABSTRACT

The proximity between microorganisms present in dental biofilm determines the existence of interaction between them, which can benefit or antagonize the involved species. The aims of this study were: 1) to present a review about interactions among cariogenic bacterial species within oral biofilm; 2) to show the applicability of the drip flow reactor (DFR) for developing oral biofilms and testing antimicrobial agents; 3) to evaluate the growth and chlorhexidine susceptibility of biofilms comprised by *Streptococcus mutans* ATCC 25175 and *Lactobacillus acidophilus* ATCC 4356, using DFR; 3) to assess the growth and chlorhexidine susceptibility of biofilms comprised by *Streptococcus mutans* ATCC 25175 and *Actinomyces naeslundii* ATCC 12104, using DFR. Biofilms grew on hydroxyapatite coated glass slides, with BHI broth at 10 mL/h flow rate supplemented with 0.2 or 0.5% sucrose depending on the species used. DFR was incubated for 24 h at 37 °C / 5% CO2. Biofilms were treated with 0.2% chlorhexidine (CHX) or 0.9% NaCl for 2 min. Growth and effect of treatments were determined by colony forming units (CFU) counts. Biofilms were stained using the Live/Dead Viability kit and analyzed by confocal laser scanning microscopy (CLSM) to differentiate bacterial cells without damage and damaged by the action of CHX. DFR validation was analyzed by unpaired t test (α=0.05). Interaction evaluation was analyzed by two-way ANOVA and Tukey test (α=0.05). Literature review showed the role of microbial interactions in balancing competition/coexistence. For DFR validation, results showed that despite distinct initial concentrations of S. mutans mono-cultures, chlorhexidine treatment affected both biofilms at the same proportion. No interaction between treatment solution and culture condition was found in *S. mutans* and *L. acidophilus* biofilms. However, viability was significantly reduced by CHX treatment. L. acidophilus in mono-culture grew significantly less than S. mutans in either mono or mixed-culture. *S. mutans* and *A. naeslundii* grew similarly in both culture conditions within NaCl group. Bacterial viability was significantly reduced in all groups treated with chlorhexidine, except for *S. mutans* in mixed-cultures. *A. naeslundii* in mono-culture was the most susceptible group, whereas S. mutans in mixed-cultures was the least susceptible. In conclusion, relationships among microorganisms may influence the occurrence of dental caries. The present study showed the applicability of the DFR for growing oral biofilms and testing antimicrobial agents. Significant interactions were found between *S. mutans* and *A. naeslundii* but not between *S. mutans* and *L. acidophilus*.

Keywords: *Streptococcus mutans*. *Lactobacillus acidophilus*. Actinomyces. Chlorhexidine. Dental caries. Biofilms.

SUMÁRIO

1 INTRODUÇÃO

Biofilmes são definidos como uma comunidade de micro-organismos metabolicamente integrada, espacialmente organizada, delimitada por uma matriz extracelular produzida pelos próprios co-habitantes (Davey[8], 2000). O processo de formação do biofilme dentário inicia-se com os colonizadores primários aderindo a película adquirida (Kolenbrander e London.[15], 1993). Eles agem como uma superfície de reconhecimento para adesão dos colonizadores secundários (Nobbs et al.[25], 2011). Diante de condições favoráveis, esses micro-organismos começam a se multiplicar, desenvolvendo uma comunidade composta por múltiplas espécies (Rickard et al.[30], 2003). A proximidade entre eles facilita a ocorrência de interações que podem tanto beneficiar quanto antagonizar os envolvidos (Marsh, Devine2[1], 2011), assim como influenciar na composição da comunidade (Kolenbrander et al.[16], 2006).

Actinomyces naeslundii é um colonizador primário do biofilme que coadere/coagrega com outras espécies, especialmente estreptococos do grupo mutans (Al-Ahmad et al.[3], 2007; Kneist et al.[14], 2012). Foi sugerido que este micro-organismo estaria associado com a baixa prevalência de cárie (Stenudd et al.[32], 2001; Levine et al.[18], 2005), devido à sua capacidade de reduzir o potencial acidogênico do biofilme através da degradação do lactato produzido por outros micro-organismos a ácidos fracos (Takahashi, Nyvad[28], 2008; Takahashi, Yamada[26], 1999). Entretanto, à medida que o biofilme se torna maduro e o meio, anaeróbio, *A. naeslundii* passa a metabolizar exclusivamente carboidratos em ácidos, cujo acúmulo ocasiona redução no pH do ambiente (Takahashi, Yamada[27], 1999). Essa queda no pH do biofilme poderia promover a proliferação de bactérias acidogênicas e ácido-tolerantes, como *Streptococcus mutans* (Takahashi, Nyvad[29], 2011). Isso explicaria a associação de espécies do gênero *Actinomyces* com o desenvolvimento de manchas brancas (Aas et al.[1], 2008) e cáries radiculares (Brailsford et al.[6], 2001).

O papel de *Streptococcus mutans* no desenvolvimento de cárie dentária, por sua vez, já está bem estabelecido (Loesche[19], 1986). *S. mutans* é capaz de metabolizar diferentes tipos de carboidratos em ácidos, desmineralizando o esmalte dentário (Moye et al.[24], 2014). Ele também produz três diferentes glicosiltransferases (Bowen, Koo[5], 2011; Koo et al.[17], 2013), relacionadas com a síntese de polissacarídeos extracelulares (Forssten et al.[9], 2010).

Lactobacillus spp. são espécies fortemente acidogênicas e capazes de sobreviver e proliferar em pH baixo (Badet et al.[4], 2008; Takahashi, Nyvad[29], 2011). Espécies desse gênero estão associadas com a progressão de lesões cariosas (Simark-Mattsson et al.[33], 2007). *L. acidophillus* pode ser encontrado tanto em cáries superficiais quanto profundas (Mei et al.[23], 2015), porém necessita de nichos retentivos para colonizar a superfície dentária (Badet et al.[4], 2008). Polissacarídeos extracelulares produzidos por outros micro-organismos, tais como *S. mutans* poderiam também melhorar a adesão de lactobacilli ao biofilme (Badet et al.[4], 2008; Wen et al.[34], 2010).

Relações de colaboração e oposição entre espécies podem influenciar a virulência e cariogenicidade do biofilme dentário (Kara et al.[12], 2006; Luppens et al.[20], 2008). Entretanto, poucos estudos foram realizados para verificar como essas interações interferem na resistência a antimicrobianos. Grande parte dos estudos in vitro disponíveis fizeram uso de sistemas estáticos para formar e tratar os biofilmes (Kara et al.[13], 2007; Guggenheim, Meier[11], 2011; Ruiz-Linares et al.[31], 2014). Entretanto, a metodologia ideal para testar a tolerância a antimicrobianos seria usar um modelo capaz de simular o ambiente no qual biofilmes são formados *in vivo*. Assim, uma abordagem eficiente seria a utilização de reatores de fluxo, nos quais biofilmes crescem sob a influência de um fluxo constante de meio de cultura (Goeres et al.[10], 2009).

Em 2002, um estudo sugeriu que o reator ‘‘drip flow’’ seria capaz de mimetizar o ambiente da cavidade bucal (Adams et al.[2], 2002), provavelmente devido à possibilidade de

gerar um fluxo lento e contínuo de meio de cultura, semelhante ao fluxo salivar na cavidade bucal. Seu uso já foi validado para o desenvolvimento de biofilmes de *Pseudomonas aeruginosa* (Method E2647-13 Standard test method[22]) e mais recentemente, o reator ‘‘drip flow’’ foi empregado para formar biofilmes de *S. mutans* e testar o potencial antimicrobiano de dentifrícios (Brambilla et al.[7], 2014). Entretanto esse modelo ainda não foi efetivamente validado para o desenvolvimento de biofilmes envolvendo micro-organismos orais utilizando um agente antimicrobiano padrão-ouro como a clorexidina.

Desse modo, os objetivos desse estudo foram: 1) apresentar uma revisão de literatura sobre interações entre bactérias cariogênicas no biofilme dentário; 2) discutir a aplicabilidade do reator ‘‘drip-flow’’ (DFR) para a formação de biofilmes dentário e teste de agentes antimicrobianos; 3) avaliar o crescimento e a suscetibilidade à clorexidina de biofilmes compostos por *Streptococccus mutans* e *Lactobacillus acidophilus*, usando DFR; 3) avaliar o crescimento e a suscetibilidade à clorexidina de biofilmes compostos por *Streptococccus mutans* e *Actinomyces naeslundii*, usando DFR.

2 PUBLICAÇÃO 1 *

Interactions among cariogenic bacterial species in oral biofilm

Rosa Virginia Dutra de Oliveira[1], **Cristiane Yumi Koga-Ito**[2], **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]. 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

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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 occur 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 seem 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 enhance *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 suggests 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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3 PUBLICAÇÃO 2*

Drip-flow reactor: a suitable approach for growing oral biofilms and testing antimicrobial agents

Oliveira RVD [1], Bonafé FSS [2], Spolidorio DMP [3], Koga-Ito CY [4], Kirker KR [5], Brighenti FL[1], Garth J [5]

[1] Department of Orthodontics and Pediatric Dentistry, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[2] Department of Social Dentistry, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[3] Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[4] Institute of Science and Technology, São José dos Campos School of Dentistry, UNESP–Univ Estadual Paulista, São José dos Campos, SP, Brazil

[5] Center for Biofilm Engineering, Montana State University, Bozeman, MT, United States

Direct correspondence and requests for reprints to Fernanda Lourenção Brighenti, Departamento de Clínica Infantil, Faculdade de Odontologia de Araraquara, Rua Humaitá, 1680, Centro, CEP 14801-903 – Araraquara-SP, phone: +55 16 33016551, fax: +55 16 33016300; Email: fbrighenti@foar.unesp.br

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/#biblioteca/manual.

ABSTRACT

The primary aim was to validate the use of drip flow reactor (DFR) to form biofilms involving oral microorganisms. A secondary aim was to use DFR to evaluate the interaction between *Streptococcus mutans* ATCC 25175 and *Lactobacillus acidophilus* ATCC 4356. For DFR validation, *S. mutans* mono-cultures at 1 x 10^2 CFU/mL and 1 x 10^4 CFU/mL were used. To evaluate the interaction between the two species, 1 x 10^4 CFU/mL for *S. mutans* and 1 x 10^8 CFU/mL for *L. acidophilus* were used to develop mono- and mixed-cultures. Biofilms grew on hydroxyapatite coated glass slides, using 0.5% sucrose BHI Broth at 10ml/h flow rate. DFR was incubated for 24 h at 37°C/5% CO_2. Biofilms were treated with 0.2% chlorhexidine (CHX) or 0.9% NaCl for 2 min. Growth and effect of the treatments were determined by CFU counts. Biofilms were stained using the Live/Dead Viability kit and analyzed by confocal laser scanning microscopy (CLSM) to differentiate bacterial cells without damage and damaged by the action of CHX. DFR validation was analyzed by unpaired t test (α=0.05). Interaction evaluation was analyzed by two-way ANOVA and Tukey test (α=0.05). For DFR validation, despite the distinct initial concentrations of *S. mutans* mono-cultures, chlorhexidine treatment affected both biofilms at the same proportion. No interaction between treatment solution and culture condition were found in *S. mutans* and *L. acidophilus*. However, viability was significantly reduced after CHX treatment. *L. acidophilus* in mono-culture grew significantly less than *S. mutans* in either mono or mixed-culture. CLSM analysis revealed a high amount of red stained cells, which suggested that bacterial cells membranes were damaged by the action of CHX. This study clearly shows the applicability of the DFR for growing dental biofilms and testing antimicrobial agents. Moreover, there was no significant interaction between *S. mutans* and *L. acidophilus*.

Keywords: *Streptococcus mutans*. *Lactobacillus acidophilus*. Biofilm. Chlorhexidine.

INTRODUCTION

The ideal methodology to test in vitro biofilm susceptibility to antimicrobials would be using a model that simulates the in vivo environment. Most of previous studies evaluated antimicrobial susceptibility using static systems to grow biofilms (Kara et al.[11], 2007; Guggenheim, Meier[9], 2011; Ruiz-Linares et al.[17], 2014). In these systems, biofilms' architecture is different because hydrodynamic stress is absent (Goeres et al.[8], 2009; Sawant et al.[18], 2013).

An effective approach to overcome this problem is the use of reactors, which avoids disadvantages related to static systems, such as bacterial sedimentation rather than attachment (Brambilla et al.[4], 2014). Moreover, the use of bacterial reactors enables the growth of relevant and repeatable biofilms under the influence of a low and constant flow of culture medium at air-liquid interface (Goeres et al.[8], 2009), which is close to that provided by saliva in oral cavity.

The drip flow reactor (DFR) was developed and validated by the Standardized Biofilm Methods Laboratory of the Center for Biofilm Engineering, for growing, treating, sampling and analyzing biofilms of *Pseudomonas aeruginosa* (Method E2647-13 Standard test method[13]). Two features of this reactor make it a good choice to mimic oral environment: a continuous low fluid shear that simulates salivary flow and clearance (Brambilla et al.[4], 2014) as well as a representative bacterial reduction observed after treatment with antimicrobial agents (Buckingham-Meyer et al.[5], 2007). To date, only one study (Brambilla et al.[4], 2014) grew *Streptococcus mutans* biofilms in DFR to evaluate the antibacterial effect of toothpastes. However, the use of this reactor has not been validated using oral bacteria and a gold standard antimicrobial agent (Twetman[23], 2004).

Substantial efforts have been dedicated to understand *S. mutans*' virulence factors, because this specie is strongly associated to dental caries. Nevertheless, species from *Lactobacillus* genus are able not only to acidify the oral biofilm, but also to survive in this in acidic environment (Badet et al.[2], 2008). *Lactobacillus acidophilus* are frequently isolated from both superficial and deep carious lesions (Mei et al.[15], 2015). However, there are evidences that lactobacilli are not able to form biofilm on their own and depend on extracellular polysaccharide produced by other microorganisms, such as streptococci (Mei et al.[14], 2013). It was also suggested that their capacity to form biofilm may be enhanced in the presence of *S. mutans* (Wen et al.[27], 2010). Thus, the study of *S. mutans* grown in mixed-cultures with *L. acidophilus* may revealed the mechanism of the interaction between them.

The primary aim of the present study was to validate and use DFR to form biofilms involving oral microorganisms. An additional aim was to use the validated model to evaluate the interaction between *S. mutans* and *L. acidophilus*. Growth and effect of the treatment were evaluated by performing viable plate counts and by analyzing the spatial arrangement of the biofilms using confocal laser scanning microscopy.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Frozen stocks of *Streptococcus mutans* ATCC 25175 and *Lactobacillus acidophilus* ATCC 4356 were inoculated, respectively, in BHI and MRS Agar (Difco, Sparks, MD, USA) and incubated for 48 h at 37 °C / 5% CO_2. Cell suspensions were prepared in saline solution (0.9% NaCl) and spectrophotometrically standardized (DU 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA, λ = 600 nm).

DFR validation

Biofilms were grown in drip flow reactor (DFR, BioSurface Technologies Inc., MT, USA). The DFR consists of a polysulfone reactor body containing six parallel channels. Each channel fits one hydroxyapatite coated glass slide (Clarkson Chromatography Products Inc., South Williamsport, PA). Thus, it is possible to run six samples per experiment. Validation of DFR use to form biofilms using oral microorganisms was initially, performed using *S. mutans* mono- cultures at 1 x 10^2 CFU/mL and 1 x 10^4 CFU/mL. For each concentration, five independent experiments were carried out.

DFR channels were inoculated with 1.0 ml of cells suspensions and incubated for 1 hour at 37 °C / 5% CO_2 with the DFRs in horizontal position. This static incubation is required to ensure bacterial attachment to the slides before the flow is initiated. Next, the reactors were inclined at 10° and medium flow was initiated at 10 ml/h rate in each channel. The medium consisted of full strength BHI broth (Difco, Sparks, MD, USA) supplemented with 0.5% sucrose.

After 24 h, each channel was rinsed with 10 ml of 0.9% NaCl to remove residual growth medium and planktonic cells. DFR was placed in horizontal position and biofilms were treated with 20 ml of 0.9% NaCl (control) or 0.2% chlorhexidine for 2 min. Chlorhexidine solution was freshly prepared on the day of use. Next, 4 h of saline solution (NaCl 0.9%) flow was used as a neutralization step.

Next, the slides were removed from the DFR and scraped thoroughly with a Teflon policeman in 10 mL of 0.9% NaCl. Slides and cell suspensions were vortexed for 30 s, sonicated for 2 min, and vortexed for an additional 30 s to remove and suspend biofilm cells. Cell suspensions were serially diluted and plated on MRS agar (Difco, Sparks, MD, USA). Plates were incubated for 48 h at 37 °C / 5% CO_2. Colony forming units were counted and the results expressed as log CFU/cm^2. Log reduction was calculated by subtracting viable cells (in log CFU/ cm^2) after treatment with chlorhexidine from viable cells (in log CFU/ cm^2) in control group.

Next, the appropriate concentration for growing *S. mutans* and *Lactobacillus acidophilus* ATCC 4356 in mixed-cultures was tested. The factor under evaluation was initial concentration of each inoculum at 2 levels, viz. 1 x 10^2 CFU/mL and 1 x 10^4 CFU/mL for *S. mutans* and 1 x 10^8 CFU/mL for *L. acidophilus.* Biofilms were grown and treated with 0.9% NaCl at the same conditions previously described. Colony forming units were counted and the results expressed as log CFU/cm^2.

Interaction between *S. mutans* and *L. acidophilus* evaluation

Three culture conditions were used: 1) *S. mutans* mono-culture, 2) *L. acidophilus* mono-culture and 3) *S. mutans* + *L. acidophilus* mixed-culture. Two treatment solutions were applied: 1) Saline solution (0.9%NaCl), as control and 2) 0.2% chlorhexidine (diacetate salt, MP Biomedicals, OH, USA). The experiment was repeated five times and thus yielded a sample size of five for each type of biofilm and treatment solution.

Mono-culture channels were inoculated with 1.0 ml of inoculum and mixed-culture channels were inoculated with 500 μL of each bacterial inoculum. Results from the pilot study determined an initial concentration of 1 x 10^2 CFU/mL for *S. mutans* and 1 x 10^8 CFU/mL for *L. acidophilus* to grow mixed-culture biofilms. Biofilms were grown, treated and analyzed at the same conditions described at "DFR validation" section. Colony forming units were counted and the results expressed as log CFU/cm^2.

Confocal laser scanning microscopy (CLSM)

Another batch of biofilms was cultivated under the same conditions described above for confocal laser scanning microscopy (CLSM) analysis. Biofilms were stained using the Live/Dead BacLight Viability kit comprising SYTO-9 and propidium iodide (Life Technologies, Oregon, USA) to differentiate bacterial cells without damage (fluorescent green) and bacterial cells with damaged membranes (fluorescent red). Images were examined using a

Leica SP5 upright confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany) with a 63× water immersion objective at 1024 x 1024 pixels resolution. Biofilms sections were obtained at 1 μm intervals in ten random positions of the glass slides. Image processing was performed using the Imaris Program (Bitplane Inc., Zurich, Switzerland).

Data analysis

For bacterial viability, statistical analysis was carried out using IBM SPSS Statistics (SPSS, Chicago, IL, USA). For DFR validation, unpaired t test was performed for bacterial viability in control group after 24 h incubation and log reduction. For the evaluation of *S. mutans* and *L. acidophilus* interaction, two factors were considered: 1) Culture condition (*S. mutans* mono-cultures, *L. acidophilus* mono-cultures and *S. mutans* + *L. acidophilus* mixed-cultures) and 2) Treatment solution (saline or chlorhexidine). Data showed equality of variances (Levene's test) and normal distribution (Kolmogorov–Smirnov test). Two-way ANOVA followed by Tukey post hoc test was performed. The significance level was set at 5% for both studies.

Confocal microscopy images were analyzed descriptively.

RESULTS

DFR validation

Table 1 shows that the higher initial concentration of *S. mutans* mono-cultures, the higher bacterial viability in biofilms treated with 0.9% NaCl ($p<0.05$). On the other hand, chlorhexidine treatment affected at the same proportion both biofilms, in despite of the distinct initial concentrations, since no statistically significant differences were observed on l log reduction.

Interaction between *S. mutans* and *L. acidophilus* evaluation

Bacterial viability was significantly reduced after chlorhexidine treatment compared with non-treated controls. Moreover, culture condition also affected bacterial viability in biofilm. However, there was no significant interaction between treatment solution and culture condition. In addition, *L. acidophilus* in mono-cultures grew significantly less than *S. mutans* in either mono and mixed-cultures (Tables 2 and 3).

Figure 1 shows confocal scanning laser microscopic images of control and treated groups of *S. mutans* and *L. acidophilus*. The increase in red stain indicates that the treatment caused an effect on bacterial cell membrane integrity. The distribution of live and dead or damaged

bacteria showed no distinct distribution pattern. The microorganisms at the biofilm surface were killed or damaged by CHX.

DISCUSSION

The present study validated a laboratory model to develop biofilm involving oral species that mimics the conditions experienced in oral cavity. Data showed that reproducible biofilms are formed in the drip flow reactor (DFR) and that the chosen experimental design was suitable. To our knowledge, this study was the first to specifically evaluate the applicability of this reactor to grow oral biofilms and to test the antimicrobial efficacy of a gold standard agent.

Oral biofilm formation and evaluation of treatment with antimicrobial agents has been performed in static systems. However, oral biofilms formed in vivo are subject to constant salivary flow, which may influence its physiological or structural properties (Blanc et al.[3], 2014). Static systems are not able to simulate these conditions. Studies have demonstrated that the biofilm structure and diffusion properties are different depending whether biofilms were formed under static or flow conditions (Buckingham-Meyer et al.[5], 2007 ; Pan et al. [16], 2010; Maezono et al.[12], 2011; Tremblay et al.[22], 2013). It is believed that the hydrodynamic stress provided by flow systems allows biofilm formation with similar features to those in vivo, regarding both architecture and susceptibility to antimicrobial agents (Goeres et al.[8], 2009; Sawant et al. [18], 2013; Blanc et al. [3], 2014). In vitro static studies usually requires a large log reduction in order to observe statistically significant differences (Guggenheim, Meier[9], 2010). On the other hand, with the reactor model used in the present study, statistically significant differences were already observed with 1 log reduction (Table 1). This indicates that DFR is a more accurate model to study oral biofilms than static systems.

No statistically significant differences in log reduction were found despite different initial concentration of *S. mutans* suspensions (1 x 10^2 CFU/mL or 1 x 10^4 CFU/mL). This indicates that bacterial viability in both biofilms was similarly affected by the antimicrobial agent applied. These results allowed validation of the proposed methodology to grow and treat biofilms involving oral bacteria.

While much effort has been devoted to understand molecular mechanisms of adherence, biofilm development and virulence gene expression by *S. mutans* in pure cultures, there are large gaps in our knowledge of how this bacterium behaves in mixed communities. The study of *S. mutans* grown in mixed-cultures with *L. acidophilus* provides means for studying a more complex microbial ecosystem and evaluating the possibility of interactions between species.

In this study, different inoculum concentrations were used for *S. mutans* and *L. acidophilus*. A higher concentration of *L. acidophilus* inoculum was required to avoid *S. mutans* overgrowth in biofilm. Interestingly, even using a six-log higher concentration of *L. acidophilus*, this bacterium showed the lowest total viability in mono-culture when compared *S. mutans* mono-culture, which had the highest total viability (Table 3). These findings may be attributed to *L. acidophilus* poor adherence properties (Mei et al. [15], 2015) and to the reason why *L. acidophilus* is associated to lesion progression (Simark-Mattsson et al. [20], 2007) instead of dental caries initiation. As a result, retentive niches (Tanzer et al.[21], 2001; Badet et al.[2], 2008) and extracellular polysaccharide produced by other microorganisms are essential to promote its colonization (Mei et al.[14], 2013). Besides, the authors observed by naked eye that *L. acidophilus* mono-cultures yielded a lower amount of biofilm than *S. mutans* mono-cultures (data not shown). This observation agrees to other studies that found a markedly thinner biofilm formed by *L. acidophilus* mono-cultures in comparison to *S. mutans* mono-cultures (Shen et al.[19], 2004; Mei et al. [14], 2013; Ahmed et al.[1], 2014).

Although it was suggested that biofilm formation by lactobacilli could be improved in the presence of *S. mutans* (Wen et al.[27], 2010), data presented in the current study did not support this finding. *L. acidophilus* populations in mixed-cultures were not significantly different from those in mono-cultures. Little data are available in the literature concerning biofilms produced by *L. acidophilus*, which hamper further comparisons.

As mentioned above, chlorhexidine is considered the gold standard antimicrobial agent in Dentistry (Twetman[23], 2004). Treatment time in the present study was increased to 2 min instead of using the standard clinical protocol (30 - 60 s) (Tomás et al. [25], 2010) because there is not the presence of oral surfaces, dental pellicle, and saliva that ensure chlorhexidine's substantivity (Hope, Wilson[10], 2004; Cousido et al.[7], 2010). In addition, 4 h of saline flow was used to better simulate the slow release of this antimicrobial agent in oral cavity (Cousido et al.[6], 2008). DFR was very useful for creating a small flow of fluid at air-liquid interface similar to that provided by saliva in oral cavity (Goeres et al [8], 2009).

It was suggested that antimicrobial efficacy tests should be performed by using laboratory methods in conditions similar to the environment where the biofilm is usually found (Buckingham-Meyer et al.[5], 2007). Thus, the present study comprehensively showed (Table 2) the anti-microbial activity of chlorhexidine against *S. mutans* and *L. acidophilus* biofilms formed and tested under ''real-use'' conditions (Goeres et al.[8], 2009).

Confocal scanning laser microscopic images showed an increase in red fluorescence after treatment with chlorhexidine was observed, indicating that this antimicrobial solution

damaged on bacterial cell membrane integrity. This is the primary effect of chlorhexidine on the bacterial structure (Vitkov et al.[26], 2005). However, the damage may occur only on the outer cell layers of the biofilm and be inadequate to induce cell death (Tomás et al.[24], 2008). Thus, Live/Dead stain provides a complementary parameter for identification of cells that possess potential to recolonize (Blanc et al.[3], 2014). On the other hand, BacLight LIVE/DEAD™ is useful to verify the spatial arrangement of the cells within the biofilm. The images presented in this study offered an overview of the amount of damaged and non-damaged cell by the antimicrobial treatment.

The observations presented here illustrate the utility of the Drip-flow Reactor for the formation of highly reproducible biofilms and emphasize the importance of studying oral biofilms formed and exposed to an antimicrobial agent in a clinically relevant environment. In this context, this study offers valuable insights about the applicability of this approach for growing and assessing treatment with antibacterial agents. In addition, our results contribute to general understanding about the susceptibility of oral bacteria within biofilms to chlorhexidine and about interaction between *S. mutans* and *L. acidophilus*.

TABLES

Table 1 Bacterial viability of *S. mutans* mono-cultures according to initial cell suspension concentration (10^2 or 10^4 CFU/mL)

Cell suspension concentration (CFU/mL)	log CFU/cm^2	log reduction
10^2	3.63±0.21[a]	1.26±0.43[a]
10^4	4.39±0.27[b]	0.84±0.47[a]

log CFU/cm^2: bacterial viability in control group after 24 h incubation; log reduction: relative number of live bacteria after chlorhexidine treatment (Unpaired t test $p<0.05$).

Fonte: Autoria própria

Table 2 Summary of two-way ANOVA results for bacterial viability

Source	df	SS	MS	F	p
Treatment solution	1	5.978	19.527	83.497	<0.001*
Culture condition	3	19.527	1.993	8.521	<0.001*
Treatment *vs* culture	3	0.680	0.227	0.969	0.419

Culture condition**	Sm mono	La mono	Sm mixed	La mixed
Sm mono	-	0.001	NS	0.047
La mono	-	-	0.002	NS
Sm mixed	-	-	-	NS

df = degrees of freedom; MS = mean square; F = MS factor/ MS residual; p = probability of significance, $\alpha=0.050$;
*statistically significant differences
**p values

Fonte: Autoria própria

Table 3 Bacterial viability (mean ± sd; Log CFU/cm^2) in mono- and mixed-cultures of *S. mutans* + *L. acidophilus*

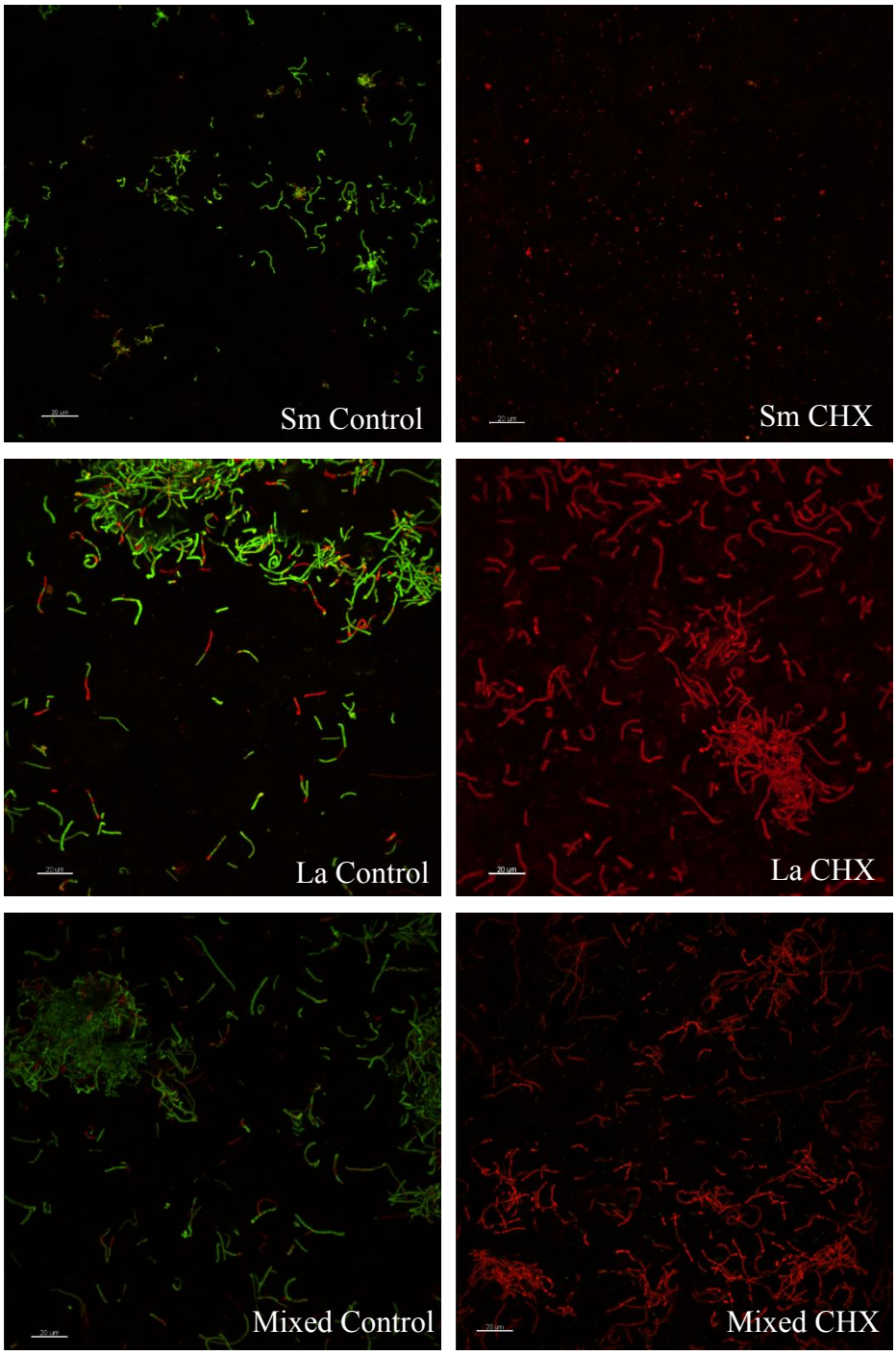
	Groups		
Species	Control	CHX	Total
La mono	2.86±0.39	1.26±0.49	2.07±0.95 [a]
La mixed	3.25±0.25	1.56±0.42	2.41±0.95 [ab]
Sm mono	3.63±0.20	2.37±0.34	3.00±0.72 [c]
Sm mixed	3.46±0.20	2.42±1.00	2.94±0.87 [bc]
Total	3.30±0.39	1.91±0.77*	2.60±0.93

La mono: *L. acidophilus* in mono-culture; La mixed: *L. acidophilus* in mixed-culture; Sm mono: *S. mutans* in mono-culture; Sm mixed: *S. mutans* in mixed-culture. *Denotes a significant difference between control and treated groups. Means followed by different lowercase letters within each row indicate statistically significant differences between biofilm type (two way Anova and Tukey test $p<0.05$).

Fonte: Autoria própria

FIGURES

Figure 1 Representative CLSM images of 0.9% NaCl (control) and 0.2% chlorhexidine (CHX) treated biofilms of *S. mutans* and *L. acidophilus* (63× water immersion).



Fonte: Autoria própria

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4 PUBLICAÇÃO 3*

A. naeslundii increases *S. mutans* resistance to chlorhexidine in mixed-culture

Oliveira RVD [1], Bonafé FSS [2], Spolidorio DMP [3], Koga-Ito CY [4], Kirker KR [5], Brighenti FL[1], Garth J [5]

[1] Department of Orthodontics and Pediatric Dentistry, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[2] Department of Social Dentistry, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[3] Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP–Univ Estadual Paulista, Araraquara, SP, Brazil

[4] Institute of Science and Technology, São José dos Campos School of Dentistry, UNESP–Univ Estadual Paulista, São José dos Campos, SP, Brazil

[5] Center for Biofilm Engineering, Montana State University, Bozeman, MT, United States

Direct correspondence and requests for reprints to Fernanda Lourenção Brighenti, Departamento de Clínica Infantil, Faculdade de Odontologia de Araraquara, Rua Humaitá, 1680, Centro, CEP 14801-903 – Araraquara-SP, phone: +55 16 33016551, fax: +55 16 33016300; Email: fbrighenti@foar.unesp.br

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ABSTRACT

The aim of this study was to evaluate the interaction between *Streptococcus mutans* ATCC 25175 and *Actinomyces naeslundii* ATCC 12104. Biofilms grew on hydroxyapatite coated glass slides placed into drip flow reactor (DFR), using 0.2% sucrose BHI Broth at 10ml/h flow rate. DFR was incubated for 24 h at 37° C / 5% CO_2. Biofilms were treated with 0.2% chlorhexidine (CHX) or 0.9% NaCl for 2 min. Growth and effect of the treatments were determined by CFU counts. Biofilms' spatial arrangement was analyzed by confocal laser scanning microscopy (CLSM). Interaction between species was analyzed by two-way ANOVA and Tukey test (ρ=0.05). A statistically significant interaction between treatment solution and culture condition was observed (p=0.001). Species grew similarly in both culture conditions within saline group. Bacterial viability was significantly reduced in all groups treated with chlorhexidine, except for *S. mutans* in mixed-cultures. *A. naeslundii* in mono-culture was the most susceptible to chlorhexidine, whereas *S. mutans* in mixed-cultures was the least susceptible. These findings suggest that the presence of *A. naeslundii* increased *S. mutans* resistance to chlorhexidine.

Keywords: Actinomyces. *Streptococcus mutans*. Biofilm. Chlorhexidine.

INTRODUCTION

Biofilm is considered a complex microbial community, whose close cell-to-cell contact results in intra- and interspecies interactions, which can benefit or antagonize the involved microorganisms (Kolenbrander et al.[11], 2006; Marsh, Devine[15], 2011).

Metabolic products of a microorganism may affect other species (Kuramitsu et al.[12], 2007). *Actinomyces naeslundii* is an early colonizer of dental biofilm, whose acid organic production is able to acidify biofilm environment and to favor the colonization of more acidogenic and acid tolerant bacteria, such as *Streptococcus mutans* [Takahashi, Yamada[19], 1999]. This observation could explain why *S. mutans* in vitro colonization was more efficient in the presence of *A. naeslundii* [Wang et al.[24], 2011]. According to Takahashi, Nyvad[21](2011), this environmental acidification may play not only phenotypic changes, but also genotypic shifts in the microorganisms, affecting the cariogenicity of the biofilm.

Additionally, interactions between biofilm species may contribute to bacterial survival and antimicrobial resistance. Previous studies have shown that interactions between *S. mutans* and *Veillonella parvula* may have been responsible for the higher survival rate after chlorhexidine exposure (Kara et al.[8], 2006; Luppens et al.[14], 2008). This outcome raises the question about how bacterial interactions in biofilm community may influence the occurrence of dental caries and antimicrobial resistance. Nevertheless, little is known about this subject.

Understanding the interactions between *S. mutans* and other oral biofilm bacteria may indicate new possibilities and strategies for dental caries prevention. A previous study from our research group has provided an insight about interaction between *S. mutans* and *A. naeslundii* (Oliveira et al.[16], 2015). In the current study, mono- and mixed-cultures of *Streptococcus mutans* and *Actinomyces naeslundii* were grown using a drip-flow reactor to examine growth and chlorhexidine susceptibility. Growth and treatment effect were evaluated by viable plate counts and an overview about biofilms' structure were obtained using confocal laser scanning microscopy analysis.

MATERIAL AND METHODS

Bacterial strains and growth conditions

An aliquot of frozen stocks of *Streptococcus mutans* ATCC 25175 and *Actinomyces naeslundii* ATCC 12104 were inoculated in BHI Agar (Difco, Sparks, MD, USA) for 48 h at 37 °C / 5% CO_2. Cell suspensions were prepared in saline solution (NaCl 0.9%) and

spectrophotometrically standardized (DU 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA, λ = 600 nm) at optical densities equivalent to approximately 1 x 10^4 CFU/mL for *S. mutans* and 1 x 10^8 CFU/mL for *A. naeslundii*. These concentrations were previously determined in pilot studies (data not shown).

Biofilm growth and treatment

Biofilms were grown in drip flow reactor (DFR, BioSurface Technologies Inc., MT, USA). The DFR consists of six parallel channels in a polysulfone reactor body. Each channel fits one hydroxyapatite coated glass slide (Clarkson Chromatography Products Inc., South Williamsport, PA). Thus, it is possible to run six samples per experiment. Three culture conditions were used: 1) *S. mutans* mono-culture, 2) *A. naeslundii* mono-culture and 3) *S. mutans* + *A. naeslundii* mixed culture). Two treatment solutions were applied: 1) Saline solution (NaCl 0.9%), as control and 2) 0.2% chlorhexidine (diacetate salt, MP Biomedicals, OH, USA). The experiment was repeated five times and thus yielded a sample size of five for each type of biofilm and treatment solution.

Single-species channels were inoculated with 1.0 ml of inoculum and dual-species with 500 μL of each bacterial inoculum. The channels were incubated at 37°C / 5% CO_2 for 1 hour with the DFRs in horizontal position. This static incubation is required to ensure that bacterial attachment to the slides before the flow is initiated. Next, the reactors were inclined at 10° and medium flow was initiated at a rate of 10 ml/h in each channel. The medium consisted of full strength BHI broth (Difco, Sparks, MD, USA) supplemented with 0.2% sucrose.

After 24 h, each channel was rinsed with 10 ml of saline solution (NaCl 0.9%) to remove residual growth medium and planktonic cells. The DFRs were placed in horizontal position and biofilms were treated with 20 ml of saline solution (NaCl 0.9%) or 0.2% chlorhexidine for 2 min. Chlorhexidine solution was freshly prepared on the day of use. Next, 4 h of saline solution (NaCl 0.9%) flow was used as a neutralization step and biofilms were analyzed.

Bacterial viability assessment

The slides were removed from the DFRs and scraped thoroughly with a Teflon policeman in 10 mL of saline. Slides and cell suspensions were vortexed for 30 s, sonicated for 2 min, and vortexed for an additional 30 s to disrupt bacterial aggregates. Cell suspensions were serially diluted and plated on BHI agar supplemented with 10% sheep blood (Difco, Sparks, MD, USA). Plates were incubated at 37 °C, for 48 h / 5% CO_2. Colony-forming units were determined and the results were expressed as Log CFU/cm^2.

Confocal laser scanning microscopy (CLSM)

Another batch of biofilms was cultivated under the same conditions described above for confocal laser scanning microscopy (CLSM) analysis. Biofilms were stained using the Live/Dead BacLight Viability kit comprising SYTO-9 and propidium iodide (Life Technologies, Oregon, USA) to differentiate bacterial cells without damage (fluorescent green) and bacterial cells with damaged membranes (fluorescent red). Images were examined using a Leica SP5 upright confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany) with a 63× water immersion objective at 1024 x 1024 pixels resolution. Biofilms sections were obtained at 1 μm intervals in z axis to obtain a three-dimensional view of biofilm in ten random positions of the glass slides. Image processing was performed using the Imaris Program (Bitplane Inc., Zurich, Switzerland).

Data analysis

For bacterial viability, statistical analysis was carried out using IBM SPSS Statistics (SPSS, Chicago, IL, USA). Two factors were considered: 1) Culture condition (*S. mutans* mono-culture, *A. naeslundii* mono-culture and *S. mutans* + *A. naeslundii* mixed culture) and 2) Treatment solution (saline or chorhexidine). Data showed equality of variances (Levene's test) and normal distribution (Kolmogorov–Smirnov test). Two-way ANOVA followed by Tukey post hoc test was performed. The significance level was set at 5%.

Confocal microscopy images were analyzed descriptively.

RESULTS

Two-way ANOVA showed a statistically significant interaction between treatment solution and culture condition (p=0.001). There were no statistically significant differences on bacterial viability treated with saline solution (control group). *S. mutans* in mixed cultures was the least susceptible species to chlorhexidine. *A. naeslundii* in mono-culture was the most susceptible species to chlorhexidine. Comparisons between treated and non-treated groups showed that bacterial viability was significantly reduced in all groups, except for *S. mutans* in mixed-cultures (Table 1).

Figure 1 shows confocal scanning laser microscopic images of control and treated groups of *S. mutans* and *A. naeslundii*. The increase in red stain indicates that the treatment caused damage on bacterial cell membrane. The distribution of live and dead or damaged bacteria

showed no distinct distribution pattern. The microorganisms at the biofilm surface were killed or damaged by CHX. Cells were evenly distributed in *A. naeslundii* mono-cultures (Fig 1). Big clusters were observed in mixed-cultures (Fig 1).

DISCUSSION

Using a laboratory model recently validated by our research group for oral biofilm formation, the present study evaluated growth and chlorhexidine susceptibility of *S. mutans* and *A. naeslundii* mono- and mixed-cultures. This model is based on growing, treating, sampling and analyzing oral biofilms developed under the influence of a constant flow of culture medium at air-liquid interface, mimicking the conditions experienced in oral cavity (capítulo 2 desta tese).

A four-log concentration of *A. naeslundii* inoculum was required to avoid *S. mutans* overgrowth in biofilm, which may be attributed to the slower cell division of *A. naeslundii* compared to streptococci (Dige et al.[5], 2009; Sánchez et al.[17], 2011). Despite this initial difference, species grew similarly within all saline-treated groups (Table 1).

The role of *A. naeslundii* on caries etiopathology is controversial. It has been suggested a correlation between the presence of *A. naeslundii* and low caries experience (Stenudd et al.[18], 2001; Levine et al.[13], 2005; Heinrich-Weltzien et al.[6], 2014). On the other hand, *A. naeslundii* may acidify the environment and favor the colonization of acidogenic and acid tolerant species, such as *S. mutans* (Takahashi, Yamada [19, 20], 1999). Moreover, it has been shown that its high acid production contributes to enamel demineralization (Kneist et al.[10], 2012) and to root caries development (Xiao et al.[22], 2012). The results of the present study corroborate with the last hypothesis and indicate that the presence of *A. naeslundii* increased *S. mutans* chlorhexidine resistance in mixed-culture biofilms.

The reduced susceptibility to antimicrobial agents of *S. mutans* in the presence of mixed cultures was previously reported by Luppens et al.[14] (2008). Some factors account for this decreased susceptibility. In general, mutans streptococci are able to coadhere with *A. naeslundii* (Al-Ahmad et al.[1], 2007; Kneist et al.[10], 2012) in a process mediated by glucosyltransferase B (Vacca, Bowen[23], 1998). This coadhesion may promote microcolonies formation, which would act as a physical barrier and affect chlorhexidine diffusion into the biofilm (Bowen, Koo[2], 2011). Furthermore, changes on bacterial virulence and gene expression (Luppens et al.[14], 2008) may have occurred. Molecular mechanisms involved in this increased resistance should be further investigated.

Whereas viable cell counts allowed quantification of cells with recolonization potential (Blanc et al.[3], 2014), the use of Live/Dead stain along with confocal scanning laser microscopic analysis provided an overview about biofilm structure. The increased red fluorescence observed in all CHX-treated groups indicates that there was damage on bacterial cell membrane. Indeed, the primary effect of chlorhexidine involves the attraction and adsorption of cationic molecules to the bacterial surface, promoting changes in cell membrane permeability, which result in irreversible loss of cellular constituents, membrane damage and enzyme inhibition [37] (Hope, Wilson[7], 2004). As previously suggested, the presence of another microorganism may influence biofilm architecture, as well as contribute to the survivability and resistance to antimicrobials (Filoche, Zhu & Wu, 2004; Kara, ten Cate and Luppens, 2006; Kara et al., 2007) [8,9,10]. *Actinomyces* spp. are early colonizers that coaggregate with mutans streptococci (Al-Ahmad et al., 2007; Kneist et al., 2012), which could be observed in CLSM images by the formation of clusters (Fig 1F). This spatial arrangement may have contributed to increased *S. mutans* resistance to chlorhexidine in mixed-culture (Kara et al.[9], 2007; Corbin et al.[4], 2011].

From the collected data, it may be concluded that inter-species interaction benefits *S. mutans* by increasing its resistance to chlorhexidine when co-cultured with *A. naeslundii.* In addition, our observations illustrate again the applicability of drip-flow reactor as tool for growing oral biofilms and test the effect of antibacterial agents in a clinically relevant environment.

TABLES

Table 1 Summary of two-way ANOVA results for bacterial viability

Source	df	SS	MS	F	p
Treatment solution	1	11.199	11.199	105.032	<0.001*
Culture condition	3	0.448	0.149	1.400	0.261
Treatment *vs* culture	3	2.191	0.730	6.851	0.001*

df = degrees of freedom; MS = mean square; F = MS factor/ MS residual; p = probability of significance, α=0.050. *statistically significant differences
Fonte: Autoria própria

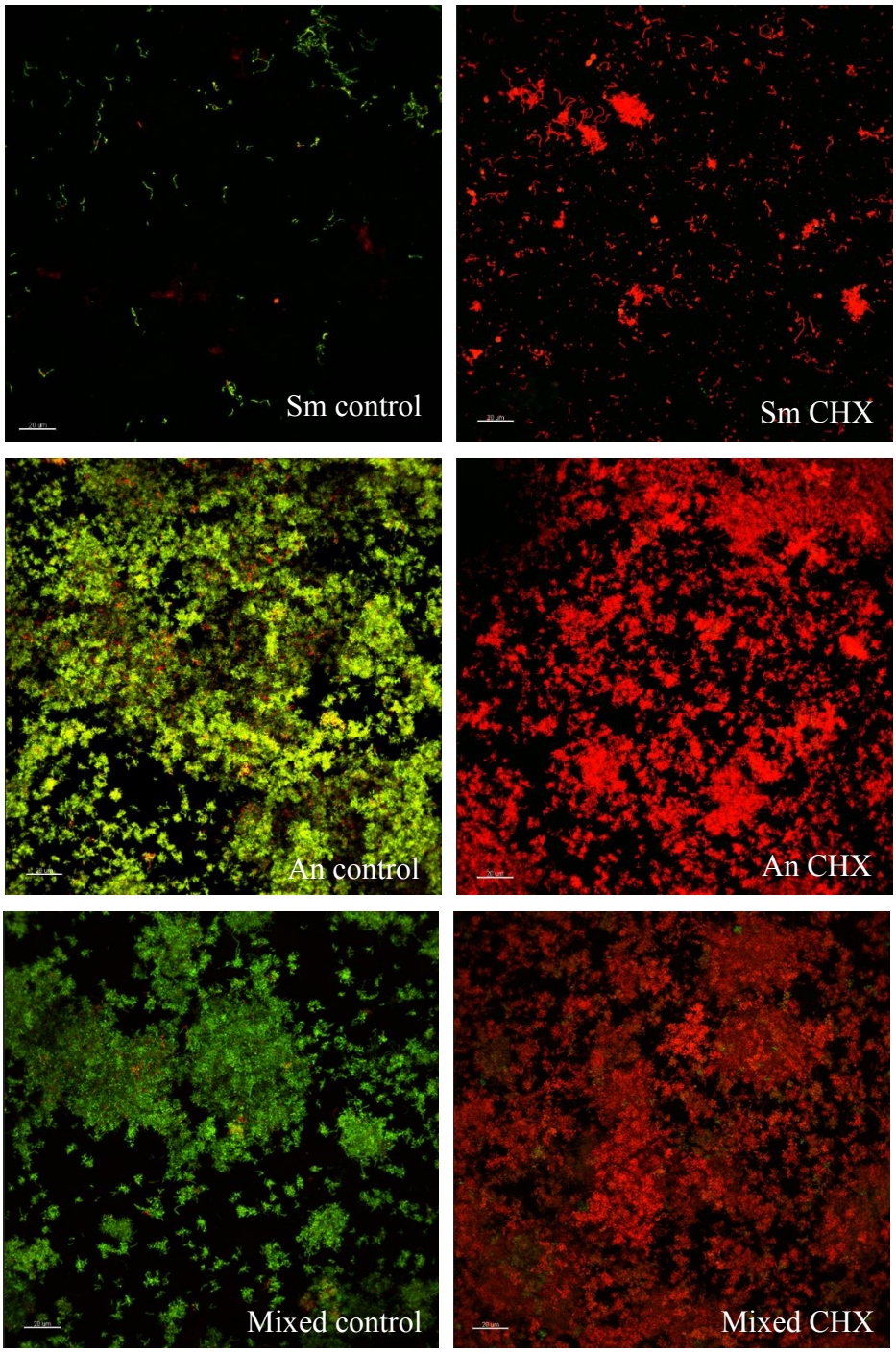
Table 2 Bacterial viability (mean ± sd; Log CFU/cm^2) in the mono and mixed-cultures of *S. mutans* and *A. naeslundii*

	Groups	
Species	Control	CHX
An mono	4.71±0.28 ^{a}	3.04±0.27 ^{B,b}
An mixed	4.59±0.14 ^{a}	3.30±0.28 ^{AB,b}
Sm mono	4.38±0.27 ^{a}	3.54±0.29 ^{AB,b}
Sm mixed	4.37±0.30 ^{a}	3.95±0.29 ^{A,a}

An mono: *A. naeslundii* in mono-culture; An mixed: *A. naeslundii* in mixed-culture; Sm mono: *S. mutans* in mono-culture; Sm mixed: *S. mutans* in mixed-culture. Means followed by different uppercase letters indicate statistically significant differences within chlorhexidine treatment. Means followed by different lowercase letters indicate statistically significant difference within each row (two way Anova and Tukey test $p<0.05$).
Fonte: Autoria própria

FIGURES

Figure 1 Representative CLSM images of 0.9% NaCl (control) and 0.2% chlorhexidine (CHX) treated biofilms of *S. mutans* and *A. naeslundii* (63× water immersion).



Fonte: Autoria própria.

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5 CONCLUSÃO

Diante dos estudos aqui apresentados, concluímos que:

- Interações entre micro-organismos podem influenciar a composição, virulência e cariogenicidade do biofilme oral, bem como a ocorrência de cárie dentária;
- O reator ‘‘drip-flow’’ demonstrou ser uma ferramenta adequada para desenvolver biofilmes orais e testar a eficácia de agentes antimicrobianos;
- Não foi observada interação significante entre *Streptococcus mutans e Lactobacillus acidophilus*
- A presença de *Actinomyces naeslundii* afetou a resistência *de Streptococcus mutans* à clorexidina.

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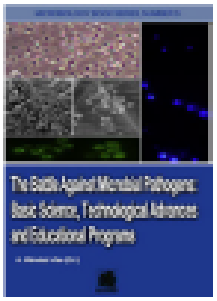
ANEXO

ANEXO 1: Autorização para publicação da Publicação 1



The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs
Microbiology Book Series – 2015 Edition

Badajoz (Spain), 13 January 2016

Dear Dr. Rosa Virginia Dutra de Oliveira,

I, Antonio Méndez-Vilas, as main editor of the fifth number of the Microbiology Book Series titled "*The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs*" and general manager of Formatex Research Center, grant you permission to use your contribution included in this research book for your own PhD. Thesis.

Thank you very much for your contribution to this book.

Best regards.

A. Méndez-Vilas
Editor for "The Battle Against Microbial Pathogens:
Basic Science, Technological Advances and Educational Programs"
Web: http://www.microbiology5.org | e-mail: book@microbiology5.org

Araraquara, 21 de março de 2016.

ROSA VIRGINIA DUTRA DE OLIVEIRA