UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Felipe Joia

Cálcio favorece a formação de biofilme por *Porphyromonas gingivalis*

Calcium increases *Porphyromonas gingivalis* biofilm formation

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Buco-Dental, na Área de concentração em Microbiologia e Imunologia.

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Orientador: Rafael Nobrega Stipp

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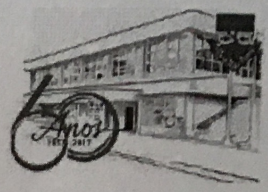

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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“Tenha coragem de seguir o que o seu coração e sua intuição dizem. Eles já sabem o que você realmente deseja. Todo resto é secundário“

“Steve Jobs“

RESUMO

A periodontite crônica é uma doença multifatorial causada por microorganismos, como *Porphyromonas gingivalis,* que afetam os tecidos de suporte dos dentes. Há poucas informações sobre as substâncias que poderiam influenciar na formação de biofilme. Por isso, o objetivo desse estudo foi avaliar a influência de proteínas, aminoácidos, elementos metálicos e vitaminas na formação do biofilme de *P. gingivalis.* O biofilme e a bactéria no estado planctônico foram cultivadas com e sem aditivos sob atmosfera anaeróbica a 37 ºC durante 18 horas. A biomassa total foi quantificada por ensaio colorimétrico e a estrutura 3D dos biofilmes foi caracterizada por microscopia confocal de varredura a laser. O ensaio de Arsenazo foi usado para quantificar a presença Ca^{+2} livre nas culturas. Um quelante extracelular foi utilizado para verificar se os reagentes testados demostram aumento do biofilme. Entre as substâncias testadas o único tratamento que resultou em um aumento significativo ($p < 0,001$ Kruscal Wallis) na formação de biofilme foi o cálcio, quando as placas foram tratadas com, pelo menos, 0,8 mg de $CaCl_2$/ cm^2. A perda da adesão bacteriana ocorreu quando um quelante foi adicionado ao meio de cultura. Os resultados sugerem que o cálcio aumenta a formação de biofilme em *P. gingivalis* sem afetar o crescimento planctônico e isso pode estar relacionado com fases iniciais do desenvolvimento do biofilme.

Palavras Chaves: Biofilme, *Porphyromonas gingivalis*, cálcio, adesão bacteriana

ABSTRACT

Chronic periodontitis is caused by microorganisms such as *Porphyromonas gingivalis* and affects the supporting tissues of the teeth. There is few information about substances which could influence biofilm formation of this bacterium. This study aim to evaluate the influence of proteins, amino acids, metallic elements, and vitamins on *P. gingivalis* biofilm formation. Biofilms were grown under anaerobic atmosphere at 37 ºC for 18 hours. A colorimetry assay (safranin) was carried out to verify the ability of the bacterium to form biofilm. A Confocal microscope was used to characterize the size and structure of the biofilm. An extracellular chelator was used to verify the effect of the test reagent showing the highest values on biofilm formation. Among the substances tested only Calcium treatment resulted in a significant increase ($p < 0.05$) of biofilm formation when plates were treated with at least 0.8 mg of $CaCl_2/cm^2$. Bacterial attachment loss occurred when a chelator was added. The findings suggest that Ca^{+2} could increase the biofilm formation. It might be related to the surface bacterial attachment and initial stages of the biofilm development.

Keywords: Calcium chloride. Biofilm development. Biofilm enhancers. *Porphyromonas gingivalis*.

SUMÁRIO

1.Introdução

1.1 Microbiota bucal

A cavidade bucal representa uma das superfícies do corpo com maior quantidade e diversidade de microrganismos (Aas et al., 2008; Kuramitsu et al., 2007; Marsh, 2003). Estima-se que mais de 1000 espécies ou filotipos microbianos habite a microbiota bucal de adultos (Dewhirst et al., 2010).

De maneira geral, a composição da microbiota bucal varia de acordo com o Habitat ou micro-habitat analisado. Como exemplo, a microbiota da saliva e de superfícies supragengivais é composta predominantemente por bactérias gram-positivas, aeróbias e anaeróbias-facultativas, como as espécies dos gêneros *Streptococcus* e *Lactobacillus*. Em contraste, a microbiota dos nichos subgengivais é composta majoritariamente por bactérias gram-negativas e anaeróbicas estritas, como as espécies dos gêneros *Campylobacter*, *Capnocytophoga*, *Eikenella*, *Fusobacterium* e *Prevotella* (Aas et al., 2005; Paster et al., 2001; Kuramitsu et al., 2007).

A microbiota bucal usualmente apresenta uma interação com o hospedeiro estável e compatível com saúde. Entretanto, episódios de desequilíbrio na composição da microbiota podem ocasionar quadros patológicos, como a cárie e as doenças periodontais, que são doenças crônicas biofilme-dependentes (Marsh & Martin, 1992; Kuramitsu et al., 2007).

1.2 Biofimes

As superfícies rígidas da cavidade bucal são colonizadas por microrganismos que crescem em biofilmes. Biofilmes são comunidades biológicas com elevado grau de organização, onde os microrganismos possuem estruturação

física e coordenação metabólica. As células encontram-se ligadas umas às outras, envolvidas por uma matriz extracelular de natureza polimérica autoproduzidas. O biofilme possui interações microbianas inter e intraespecíficas e a matriz extracelular do biofilme apresenta canais, por onde atravessam fluídos contendo nutrientes, metabólitos secretados, enzimas, oxigênio, sais e compostos orgânicos (Garrett et al. 2008). A associação das células em biofilmes constitui uma forma vantajosa de desenvolvimento que permite benefícios reprodutivos e metabólicos, além de defensivos aos outros organismos, às substâncias e à defesa do hospedeiro (Epstein et al., 2011, Renner & Weibel, 2011; Huang et al., 2011, Dentino et al., 2013).

A formação do biofilme é didaticamente dividida em quatro etapas principais: (1) adesão à superfície, (2) produção da matriz extracelular, (3) formação de micro colônias e intensificação da produção da matriz extracelular e (4) maturação e dispersão (Renner & Weibel, 2011; Abdalla et al., 2015).

Nas superfícies dentais, o processo de formação dos biofilmes tem início sobre a película adquirida - uma fina camada adsorvida aos minerais dentais. As espécies colonizadoras iniciais possuem a capacidade de interagir com componentes e proteínas da película adquirida e iniciam a colonização através de ligações não covalentes fracas, como as ligações de Van Der Waals, interações hidrofóbicas, pontes de hidrogênio e pontes iônicas (Jakubovics & Kolenbrander, 2010; Huang et al., 2011).

As principais espécies colonizadoras primárias orais são *Streptococcus gordonii, Streptococcus oralis, Streptococcus mitis e Streptococcus sanguinis.* Esses microrganismos possuem componentes proteicos em sua parede celular que permitem a adesão subsequente de espécies intermediárias como, por exemplo, *A.*

naeslundii e Fusobacterium nucleatum, que por si possuem diminuída afinidade de adesão à película adquirida (Kolenbrander. et al., 2002). Durante o estabelecimento dos colonizadores primários e tardios há a produção da matriz extracelular polimérica que compõe o biofilme e facilita a coadesão e coagregação microbiana (Kolenbrander. et al., 2002).

Pelos mecanismos descritos, o biofilme dental passa por sucessão ecológica de espécies, acompanhado de aumentos no volume e na complexidade metabólica, até chegar em estágio de maturação, o qual pode favorecer o desenvolvimento de patologias. Alterações no biofilme classicamente associadas ao favorecimento de espécies patógenas e biofilme periodonto patogenio que incluem, por exemplo, a diminuição da concentração de oxigênio e a acidificação (Huang et al., 2011; Teles et al., 2013).

1.3 Periodontite e *Porphyromonas gingivalis*

As periodontites são doenças periodontais inflamatórias que acometem os tecidos de suporte e de sustentação dos dentes. Afetam cerca de 743 milhões de pessoas e um dos tipos mais prevalentes é a periodontite crônica (Richards, 2013). A *Porphyromonas gingivalis* é uma das espécies mais associadas com o início e a progressão de quadros inflamatório no periodonto (van Winkelhoff et al., 1988).

Porphyromonas gingivalis é uma espécie Gram-negativa, anaeróbia estrita, assacarolítica, não motilizada e que forma colônias pigmentadas em preto (Shah & Gharbia, 1992; Socransky et al., 1998). Sua participação na iniciação e na progressão das doenças periodontais é favorecida por diversos fatores de virulência como adesinas, fímbrias, cápsula, lipopolissacarídeos, ácidos lipoteicóicos, gingipaínas, hemaglutininas, proteínas da membrana externa e vesículas da

membrana externa; e grande capacidade de invasão de células eucarióticas (Shibata et al., 1993; Jansen et al., 1995; Dorn et al., 1998; Socransky et al., 1998; Chen et al., 1999; Bostanci & Belibasakis 2012; Sakanaka et al., 2016).

No biofilme subgengival, *P. gingivalis* atua como um colonizador tardio, um processo facilitado por microrganismos como *Fusobacterium nucleatum* e outros colonizadores antecessores. Quando presente no biofilme, mesmo em baixas concentrações, possui a capacidade de elevar a virulência do biofilme periodontal, modular a resposta do hospedeiro, alterar a estrutura do biofilme e aumentar a carga bacteriana total, contribuindo para a patogenicidade do biofilme (Sakanaka 2016).

1.4 Estudos com biofilmes

Por muito tempo os estudos com microrganismos foram realizados com crescimento livre em meios de cultura líquidos. Entretanto, o comportamento dos microrganismos quando em crescimento planctônico é totalmente distinto daquele quando crescidos em biofilmes (Kuramitsu et al., 2007). Além disso, é estimado que cerca de 99% dos microrganismos cresçam como biofilmes em seus habitats, enquanto que apenas 1% exista na forma planctônica (Mendez-Vilas, 2011).

A reprodução exata de um biofilme dental *in vitro* é teoricamente impossível pela quantidade de microrganismos envolvidos e condições ambientais únicas (Blanc et al., 2014). Mesmo modelos multiespécies apresentam várias limitações, sendo a falta de reprodutibilidade a principal (Tang et al., 2003). Dessa forma, no nível de conhecimento atual, a maior parte dos estudos é realizada com modelos de biofilmes monoespécies, que embora simplistas, permitem a investigação científica de forma controlada e reprodutível *in vitro* (Pamp et al., 2009).

A obtenção de biofilmes monoespécies é relativamente fácil com espécies colonizadoras primárias (Jakubovics & Kolenbrander, 2010; Huang et al., 2011). Entretanto, espécies colonizadoras tardias, como *P. gingivalis*, que não se ligam diretamente ao substrato e sim aos colonizadores primários, podem apresentar limitada capacidade de formação de biofilme monoespécie (Bao et al., 2014).

Para contornar essa limitação, têm se testado a suplementação do meio de cultivo com aditivos, tais como Cálcio, Magnésio, Sacarose e outros que podem intensificar a capacidade da aderência celular e da consequente formação do biofilme (Sauer et al., 2002; Hinsa et al., 2003; Patrauchan et al., 2005; Sarkisova et al., 2005; Hansch & Mendel, 2009; Cruz et al., 2012). Cátions como, por exemplo, Ca^{+2} e Mg^{+2} podem favorecer a formação do biofilme por intensificar as interações eletrostáticas ou modular processos fisiológicos, como a produção de cápsula (Patrauchan et al., 2005; Song & Leff, 2006, Bao et al., 2014).

Em *Streptococcus mutans*, o crescimento em biofilmes monoespécie *in vitro* é estimulado pela presença e quantidade de sacarose, enquanto que em *Streptococcus sanguinis*, por superfícies tratadas com saliva (Nobbs et al., 2008). O Ca^{+2} é um importante fator para a formação de biofilme em diversas espécies bacterianas como: *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudoalteromonas* sp., *Vibrio cholerae*, *Staphylococcus aureus*, *Xylella fastidiosa* e *Citrobacter werkmanii* (Koerstgens et al., 2001; Sarkisova et al., 2005; Cruz et al., 2012; Shukla et al., 2013; Kannan et al., 2014; Y. Li et al., 2014).

Entretanto, não se conhece estímulos laboratoriais que favoreçam a formação de biofilmes monoespécie robustos *in vitro* por *P. gingivalis.*

Os objetivos desse trabalho foram:

- Encontrar substâncias que aumentam a formação de biofilmes monoespécie por *P. gingivalis,* e

- Quantificar e caracterizar a estrutura do biofilme obitido.

Calcium increases *Porphyromonas gingivalis* biofilm formation

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Abstract

Porphyromonas gingivalis is involved in the pathogenesis of periodontitis and in other common diseases in humans. AIM: In this study, we evaluate substances and fluids that may trigger *P. gingivalis* cell adhesion and biofilm formation, events that are usually required for a successful infection. Polystyrene surfaces were coated with seventeen substances or fluids and biofilm mass was quantified by spectrophotometry. Biofilm architecture and viability were analyzed by Confocal Laser Scanning Microscopy. RESULTS: Surfaces coated with 0.8 mg/cm^2 of $CaCl_2$ increased 10-fold biofilm formation (p<0.01, ANOVA), while other substances had modest or no impact in the biofilm amount. Biofilms obtained are firmly attached and structures archived height up to 120 μm, with no visible dead cells. CONCLUSION: Calcium chloride strongly increase the formation of *P. gingivalis* viable biofilms, which could be used in further studies aimed at this pattern of growth.

Keywords: Calcium chloride. Biofilm development. Biofilm enhancers. *Porphyromonas gingivalis.*

Introduction

The Gram-negative *Porphyromonas gingivalis* is a major etiological agent involved in the initiation and progression of periodontitis, one of the most common diseases of the oral cavity that affects 743 million people worldwide (Richards, 2013). Also, the specie is a potential mediator in the etiology of a variety of presumably unrelated chronic diseases, such as rheumatoid arthritis, cardiovascular diseases, diabetes and orodigestive cancers (Atanasova and Yilmaz, 2014). Although the mechanisms of pathogenesis by *P. gingivalis* still unclear, release of bacterial products, modulation of the host immunological response and biofilm formation are determinants for diseases development (Sakanaka, 2016). Once present, even in low concentrations, *P. ginvivalis* can increase the total bacterial load and the virulence of biofilms (Sakanaka, 2016).

Biofilms consists of many cells co-adhered by means of physical appendages and extra-cellular polymeric substances (reviewed in Garrett et al. 2008). *P. gingivalis* possess various surface-associated proteins that favors the biofilm formation and cell attachment. These include fimbrillins (Mfa1, FimA, FimR and FimS) and adhesins-like proteins (RgpA, RgpB, Kgp) (Lo et al., 2010; Ikai et al., 2015).

In oral biofilms, *P. gingivalis* has been shown to be a late colonizer, an event that is facilitated by species like *Fusobacterium nucleatum* and other intermediate colonizers (Sakanaka, 2016). Usually, oral late colonizers do not form robust *in vitro* monospecies biofilms, which restrict the studies and knowledge about their biofilm-associated physiology, like growth factors, dose-response to antimicrobials and gene expression analysis (Lebeaux et. al., 2013).

Various approaches have been suggested to enhance the *in vitro* biofilm formation by non-early colonizers. These include the addition of salts and/or

minerals, which may modify electrostatic interactions between cells and substrate, or physiological processes inductors, such as nutrients and enzyme cofactors (Rose, 2000; Chen & Stewart 2002; Pamp et al., 2009; Cruz et al., 2011, Lebeaux et. al., 2013).

In this study, we evaluated substances and fluids that could induce *P. gingivalis* cell adhesion, biofilm formation and analyzed the architecture, viability and coverage of the biofilms achieved.

Materials and Methods

Bacterial strain and culturing

P. gingivalis W83 (ATCC® BAA-308™) was grown anaerobically on TSB-BHI Blood Agar supplemented with Hemin and Vitamin K1, at 37 °C in anaerobiosis (80% N_2, 10% H_2, 10% CO_2) for 72 h (MiniMacs Anaerobic Workstation, Don Whitley Scientific, Shipley, UK). Isolated colonies were inoculated into 5 ml of TSB-BHI broth medium, prepared with 1.55% Tryptic Soy Broth (Difco Co., Detroit, MI, USA), 1.48% Brain Heart Infusion (Difco), 0.2% Yeast Extract (Difco), 5 µg/ml hemin and 1 µg/ml menadione (Shelburn et al., 2008), and grew until cultive reach the absorbance (A_{550nm}) of 1.2 (about 18 h).

Compounds and plate coating

Substances and fluids evaluated as potential biofilm enhancers are listed in Table 1. Fluids were used as is, and powders were solubilized using ultrapure water and filter-sterilized at 0.22 µm.

The bottom surfaces of the wells (flat bottom, 48-wells polystyrene microplates, Costar #3548, Corning Life Sciences, Acton, MA, USA) were rinsed with 100 µl of substances or fluids (Table 1) and let to dry overnight at 60°C for coating.

The resultant mass coated on the surfaces ranged from 0.005 mg/cm^2 to 2.5 mg/cm^2. Calculated area of bottom surface of the well is equal to 1 cm^2.

Table 1: Substances and fluids evaluated as coating for biofilm formation by *P. gingivalis*

Type	Coating substance or fluid	amount evaluated
Metallic salts	Cálcio chloride ($CaCl_2$)	0.125 to 2.0 mg/cm^2
	Potassium chloride (KCl)	0.125 to 0.8 mg/cm^2
	Calcium carbonate ($CaCO_3$)	0.125 to 0.8 mg/cm^2
	Calcium Hydroxide ($CaOH_2$)	0.125 to 0.8 mg/cm^2
	Magnesium chloride ($MgCl_2$)	0.125 to 0.8 mg/cm^2
Proteins	Mucin	0.125 to 0.8 mg/cm^2
	Bovine Serum Albumin	0.125 to 0.8 mg/cm^2
Amino acids	Cysteine ($C_3H_7NO_2S$)	0.125 to 0.4 mg/cm^2
	Glutamine ($C_5H_{10}N_2O_3$)	0.125 to 0.4 mg/cm^2
	Aspartic acid ($C_4H_7NO_4$)	0.125 to 0.4 mg/cm^2
	Methionine ($C_5H_{11}No_2S$)	0.125 to 0.4 mg/cm^2
Vitamins	Riboflavin ($C_{17}H_{20}N_4O_6$)	0.125 to 0.8 mg/cm^2
Sugar	Glucose ($C_6H_{12}O_6$)	0.125 to 0.8 mg/cm^2
Fluids	Defibrinated sheep blood	100 µl/well
	Horse serum	100 µl/well
	Human saliva	100 µl/well
	Artificial saliva (Roberts. et al., 2013)	100 µl/well

Biofilm quantification

The biofilm formation by *P. gingivalis* W83 was measured according to Cruz et al. (2012), with some modifications. Grew cultures were diluted to A_{550nm} of 0.3 in fresh media and 1 ml transferred to coated wells. After 24 h of incubation, media was removed and wells were washed with 1 ml of 0.9% NaCl. Biofilms were stained with 200 µl 0.1% safranin for 1 min and plates were washed three times by submersion in distilled water. Dye was eluted from biofilms with 500 µl of 95% ethanol and 80 µl of

10% SDS for 5 min and 200 µl of the homogenate transferred to 96-well plate and measured by absorbance (A_{600nm}).

Planktonic growth

To ensure the higher biofilm formation are not related to micronutrient repletion (i.e. in absence of substances the media was acting as a growth limiter), the effect of the substances and fluids in planktonic growth was evaluated. Glass tubes containing 4 mL of TSB-BHI with *P. gingivalis* (A_{550nm} = 0.3) received same amounts of substances or fluids and were incubated for 24 h. Tubes were vortexed and total bacterial growth was measured by A_{550nm}.

Structure *of P. gingivalis* biofilm

The biofilm structure was analyzed by Confocal Laser Microscopy Scanning using glass slides (Lab-Tek Chamber SlideTM #177399; Nalge Nunc International, Naperville, IL, USA) coated with $CaCl_2$ (0.8 mg/cm2). Chambers were incubated with 1 ml of TSB-BHI with *P. gingivalis* (A_{550nm} = 0.3) for 24 h and wells were washed with 0.9% NaCl. Biofilms were stained with 500 µl of BacLight Live/Dead (Life Technologies Corporation, Carlsbad, CA, USA), accordingly with manufacturer instructions. Dyes were excited at 488 nm and Z-stack images were captured with 1 µm increments under a 20x objective (LEICA TCS SP5 Microscope, Leica Microsystems, Wetzlar, Germany). Three-dimensional reconstruction, coverage and viability were assessed with Fiji software, version 2.0.0-rc-54/1.51i (Fiji, ImageJ, Wayne Rasband National Institutes of Health).

Calcium quantification

Arsenazo III were used for estimating calcium amount in media, accordingly to Leitão et al., 2012, with minor modifications. Briefly, 25 µl of cell-free culture supernatants were mixed with 125 µl of ultrapure H_2O and 150 µl of 1 mM Arsenazo III (Sigma-Aldrich Co., St. Louis, MO, USA) in a microplate. Reactions were read at 650 nm. A standard curve ranging from 0.4 mM to 0.004 mM $CaCl_2$ diluted in fresh media were used as reference.

Supplementation of media with chelator

To confirm that increase in the biofilm formation is a direct effect of $CaCl_2$, assays with the extracellular calcium chelator, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), were conducted. EGTA (#03779 Sigma-Aldrich) was solubilized in ultrapure water at concentrations 3.2 times higher than those of $CaCl_2$ and filter sterilized (Cruz et al. 2012). EGTA was used either soluble or coated within $CaCl_2$.

Transmission electron microscopy

P. gingivalis cells from a biofilm formed on a non-coated surface (control) and B) *P. gingivalis* cells from a biofilm formed on a $CaCl_2$ (0.8 mg/cm^2) were collected by micropipette aspiration and subjected to Transmission Electron Microscopy. Bacterial cells were fixed with Karnovsky solution containing 1% Alcian Blue 8GX and incubated for 4 h at 4ºC. Cells were washed three times with Sorensen phosphate buffer, stained with 1% osmium tetroxide for 90 min and dehydrated through a ten-graded series from 30 to 100% acetone. Samples were sequentially incubated with Dr. Spurr resin-acetone mixtures (3:1 for 4 h, 1:1 for 18 h, 1:3 for 3 h) and pure resin

for 1 h, and polymerized for 48 h at 60ºC. The blocks were trimmed at 70-nm ultrathin sections (Leica EM UC6, Leica Microsystems, Mannheim, Germany). The sections were collected on 200-mesh former carbon-coated copper grids and stained with 2% uranyl acetate and then Reynolds' lead citrate in a CO_2 free atmosphere and washed.

Air dried samples were evaluated with a Jeol 1400 transmission electron microscope at 80 kV (JEOL, Tokyo, Japan).

Statistical analyses.

All experiments were performed using at least six replicates per group and in at least four separate occasions. Data distribution was evaluated by Shapiro-Wilks and those with a normal distribution were compared by ANOVA and Bonferroni or Dunnett *post-hoc* test. Non-normally distributed data were analyzed by Kruskal-Wallis and Dunn *post-hoc* test. The significance level was set below 5%.

Results

$CaCl_2$ increase biofilm formation by *P. gingivalis* W83

Among substances and fluids evaluated, $CaCl_2$ was the only one that significantly increased biofilm formation by *P. gingivalis* (Figure 1). At 0.4 mg/cm^2, $CaCl_2$ increased by 10-fold compared to the control. At 0.8 mg/cm^2, the increase by $CaCl_2$ was 14-fold. Biomass was not increased in higher coated $CaCl_2$ concentrations a plateau of biofilm formation was observed. Then, a concentration of $CaCl_2$ at 0.8 mg/cm^2 was considered as optimum for the biofilm formation by *P. gingivalis.* Blood and serum did not induce biofilm formation (data not show); these stained themselves and left some absorbance reads in the assay.

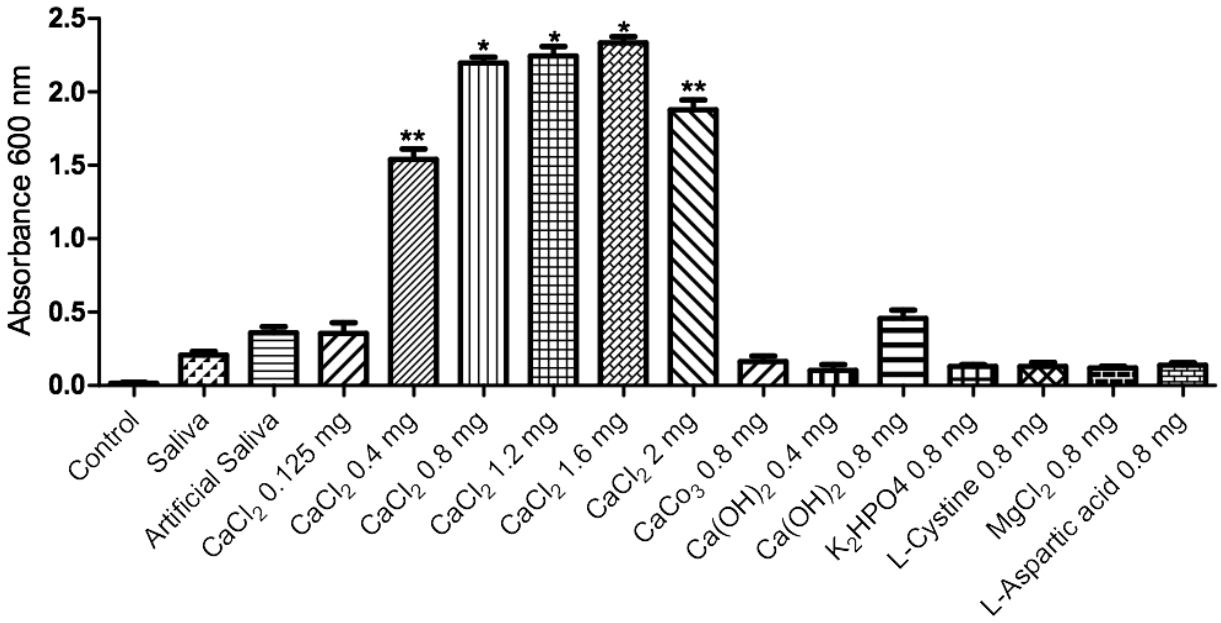

Figure 1. Mean and standard deviation of biofilms formed by *P. gingivalis* W83. Substances and fluids were surface-coated on the microplates wells and biofilms allowed to form on their tops. Control: biofilm formed on polystyrene surface without coating. Substances and fluid with absorbance (A_{600nm} above the value of 0.1 are showed. ** $p<0.05$ (ANOVA Dunnett) and * $p<0.01$ (ANOVA Dunnett).

Biofilms obtained at coated $CaCl_2$ (0.8 mg/cm^2) are firmly attached and structures archived height up to 120 µm, with no visible dead cells or clusters. The images obtained by the stereoscopic microscope (Figure 2) confirmed an increase in biofilm formation in the presence of $CaCl_2$ (0.8 mg/cm^2).

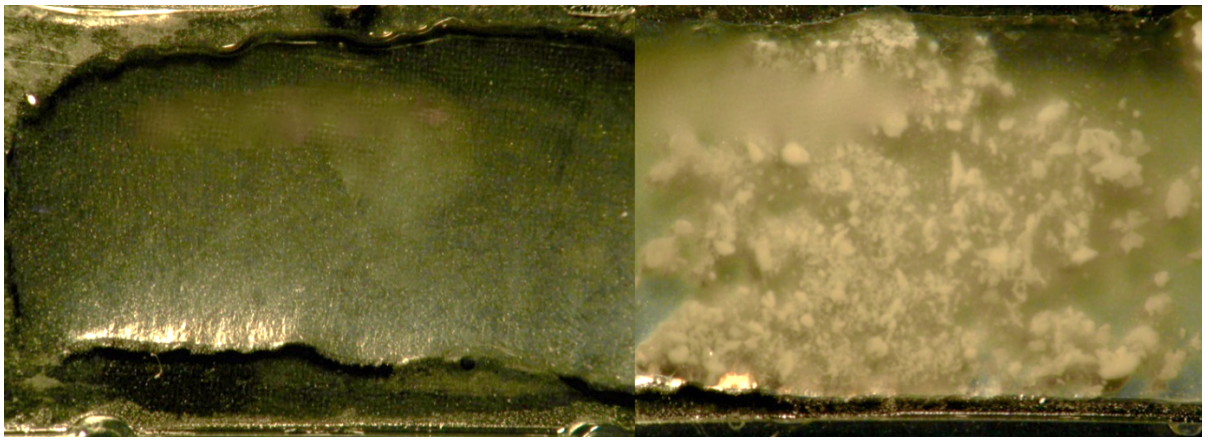

Figure 2. Photos of *P. gingivalis* W83 biofilm formed on Lab-Tek slides (Microscope stereoscopy 32x). (A) surface without coating and (B) surface coated with $CaCl_2$ at 0.8 mg/cm^2.

$CaCl_2$ does not increase the planktonic growth of *P. gingivalis* W83

The robust biofilm growth after addition of $CaCl_2$ could firstly indicate a depletion of this micronutrient in the non-supplemented culture medium. As show in Figure 3, $CaCl_2$, and other substances, did not increased yield of *P. gingivalis* after 24 h of growth when compared to equivalent culture medium (with no supplementation). This indicate that culture medium fulfils bacterial needs and no Ca^{+2} depletion occurs (Figure 3). The overall obtained A_{550nm} of 1.2 for growth cultures could be considered as optimal (Figure 3). The addition of 0.8 mg $Ca(OH)_2$ decreased the overall growth compared to the control, probably due to the pH change.

Available calcium in the medium was calculated between sterile medium against cell removed used medium after 24 h of growth. The results showed *P. gingivalis* uses about 11.8 % (± 5 %) of Ca^{+2} and the amount of Ca^{+2} is should not be a limiting factor for *P. gingivalis* growth in this medium.

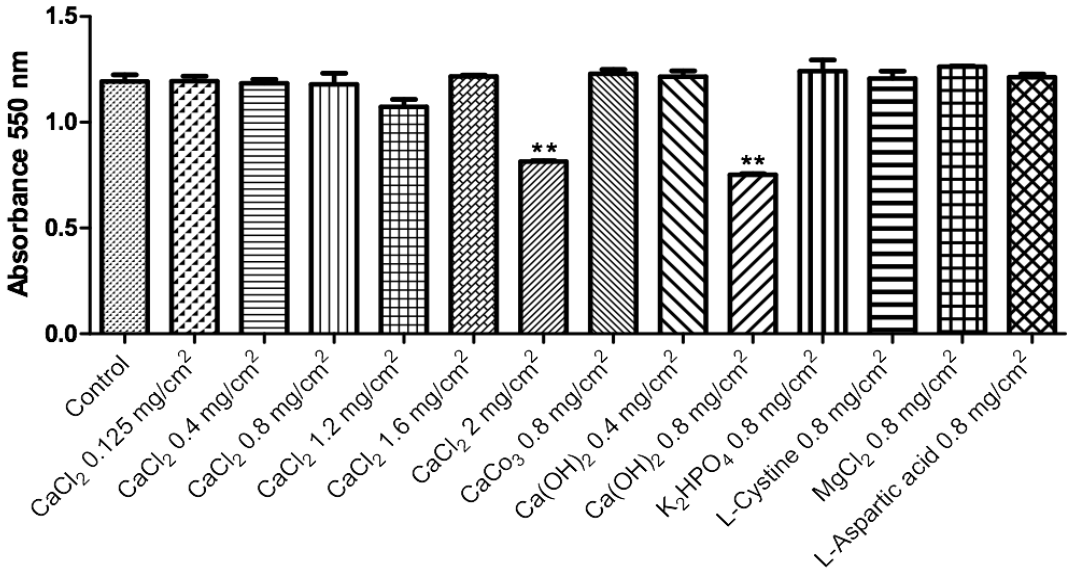

Figure 3. Measurements of the planktonic growth of *P. gingivalis* W83 after 24 h of growth. Control: TSB-BHI without supplementation. (** $p<0.05$; Kruskal-Wallis).

EGTA inhibits biofilm formation by *P. gingivalis* in $CaCl_2$ coated surfaces

Figure 4 shows biofilm formation by *P. gingivalis* in conditions of non-supplemented culture medium (control), $CaCl_2$ added in the medium (soluble $CaCl_2$) coated $CaCl_2$ and coated $CaCl_2$ plus EGTA (at 3.2x concentration of $CaCl_2$). $CaCl_2$ only plays a role in the biofilm formation when coated on the surface. The EGTA, an extra-cellular calcium chelator, inhibited the biofilm formation on presence of coated $CaCl_2$ (Figure 4).

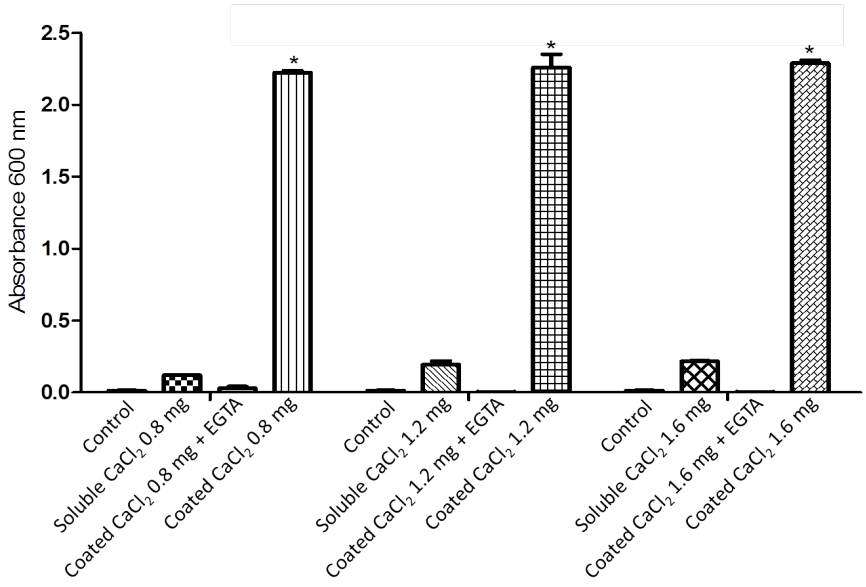

Figure 4. Mean and standard deviation of biofilms formed by *P. gingivalis* W83. Substances and fluids were surface-coated on the microplates wells and biofilms allowed to form on their tops. Control: biofilm formed on polystyrene surface without coating. * $p<0.01$ (ANOVA Dunnett).

Three-dimensional structure of biofilm formed with $CaCl_2$ at 0.8 mg/cm^2

The imagens obtained by CLSM is represented by Figure 5. Control exhibit about 5.8 % (± 8.4 %) of *P. gingivalis* W83 surface coverage, while biofilms with coated $CaCl_2$ at 0.8 mg/cm^2 showed a surface coverage of 57.0 % (± 15.7 %). Biofilms formed with $CaCl_2$ above 0.4 mg/cm^2 concentrations arrived up to 120 micrometers and were firmly attached (data not showed). Regardless of the concentrations of the $CaCl_2$ used, biofilms presented extensive areas of cell viability,

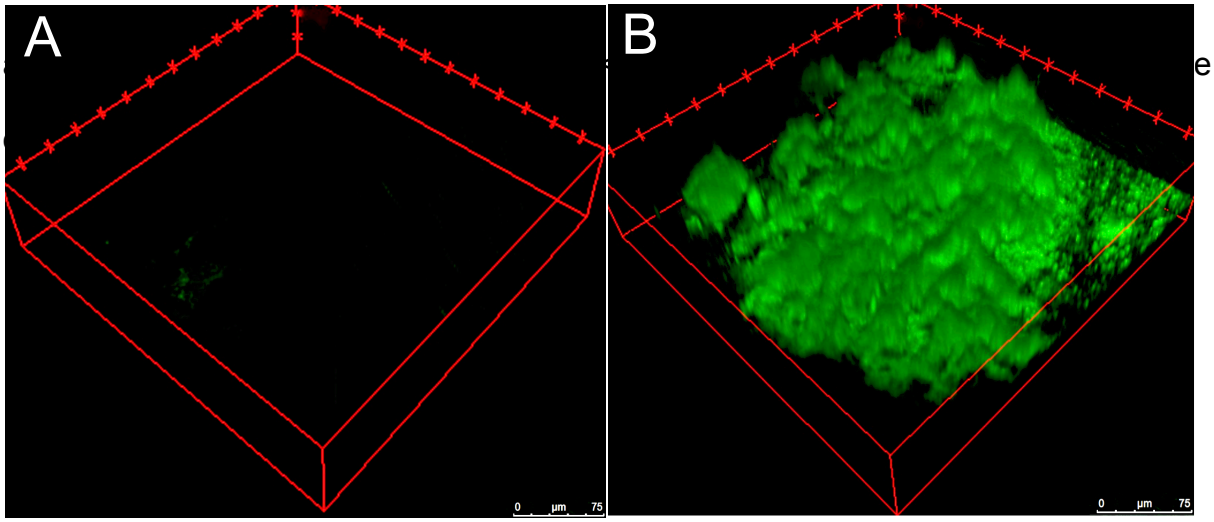

Figure 5. Images of *P. gingivalis* W83 biofilm formed on Lab-Tek slides visualized by CLSM. (A) surface without coating (control) and (B) surface coated with $CaCl_2$ at 0.8 mg/cm^2.

Capsule production is lower on surfaces coated with $CaCl_2$

The imagens obtained by TEM (transmission electron microscope) is represented by Figure 6. Control exhibit *P. gingivalis* W83 with capsule formed, while in biofilms formed in $CaCl_2$ (coated, 0.8 mg/cm^2), lower amount of capsule around the cells.

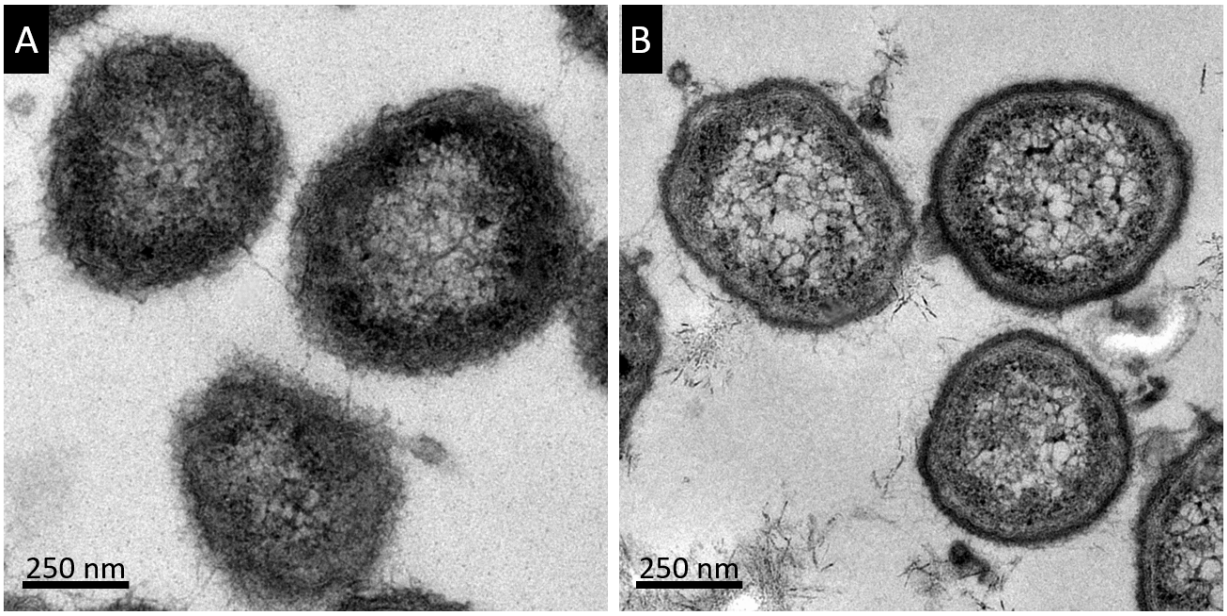

Figure 6. A) *P. gingivalis* cells from a biofilm formed on a non-coated surface, with capsule formation (control) and B) *P. gingivalis* cells from a biofilm formed on a $CaCl_2$ (0.8 mg/cm^2) coated surface, showing lower amount of capsule.

Discussion

The microorganisms are rarely found in the environment in planktonic form, but in the form of complex biofilm communities (Flemming & Wingender, 2010; Renner & Weibel, 2011). To reproduce equivalent conditions *in vitro* still a challenge for the Medical field. Several factors may affect the *in vitro* biofilm formation, such as media composition, interaction with other microorganisms, adhesion surface, temperature, pH and others (Song & Leff, 2006). In this study, we evaluated different substances and fluids that could contribute to the formation of *in vitro* mono-species biofilm of *P. gingivalis*, a late colonizer of subgingival biofilms associated with periodontal diseases (Lamont & Jenkison 1998; Griffen et al 1998; Nelson et al 2003). Among the substances and fluids tested, it was verified that $CaCl_2$ showed is an adequate surface inducer, being able to stimulate the formation of robust biofilms of *P. gingivalis*.

Cations such as Mg^{+2} and Ca^{+2}, may influence biofilm formation directly through electrostatic interactions, or indirectly influencing physiological processes as enzymatic cofactors (Patrauchan et al., 2005; Song & Leff, 2006). Calcium has been considered an important factor for biofilm formation in other species, being involved in specific and non-specific interactions between the cells and the substrate (Koerstgens et al., 2001; Patrauchan et al., 2005; Das et al., 2014). In our study, $CaCl_2$ played a role in the adhesion and formation of a robust biofilm of *P. gingivalis*, especially in the concentrations: 0.8 mg/cm^2; 1.2 mg/cm^2 and 1.6 mg/cm^2. This effect of $CaCl_2$ could be seen in the absorbance readings by the colorimetric method and visually by the images obtained.

The differences observed in the formation of biofilm in the presence of $CaCl_2$ led to two hypotheses: if the absence of biofilm formation could be related to a

restriction of Ca^{+2} in the culture medium; or if calcium could stimulate total bacterial growth, leading to an increase of biofilm formation. However, the supplemented planktonic assay showed that $CaCl_2$ does not increase the cell growth of *P. gingivalis*. Furthermore, quantification of Ca^{+2} in the culture medium and in the control showed that *P. gingivalis* does not deplete Ca^{+2} once available in formerly culture medium. Therefore, biofilm formation of *P. gingivalis* in the presence of $CaCl_2$ is not related to metabolism of Ca^{+2} and growth stimulus.

When $CaCl_2$ is added directly in to the culture medium the biofilm formation was no statistically significant. In contrast, when $CaCl_2$ is coated to the surface, the process of adhesion and biofilm formation occurs. And then, when a calcium chelator (EGTA) is added to the coated $CaCl_2$, biofilm formation by *P. gingivalis* is inhibited and the structure of the biofilm is absent.

The application of Confocal Scanning Laser Microscopy (CSLM) to biofilm research radically altered the perception of biofilm structure and function, and allowing structure analysis and cellular viability quantification (Drago et al., 2015). *P. gingivalis* biofilm structure analysis demonstrated a biofilm with characteristics that are typically associated with a mature biofilm (Donlan, 2002). The images showed a heterogeneous architecture consisting of large macro-colonies surrounded by open areas on the surfaces (Donlan, 2002). Also, the thickness of the biofilm was up to 120 μm, which is comparable to measurements obtained in studies on other model organisms, such as *Pseudomonas aeruginosa, and Staphylococcus aureus* (Valeria et al., 2014).

The ability of calcium to influence the formation and architecture of the biofilm has already been described for microorganisms such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas* sp., *Vibrio cholerae*, *Staphylococcus*

aureus, *Xylella fastidiosa* and *Citrobacter werkmanii* (Koerstgens et al., 2001; Sarkisova et al., 2005; Cruz et al., 2012; Shukla et al., 2013). Among the possible mechanisms of action described in the literature that could explain the participation of calcium in the biofilm, we have: a higher production of extracellular matrix, promotion of cellular aggregation, signaling of gene expression for components related to biofilm, electrostatic interaction between the extracellular matrix and on the substrate, and the binding of Ca^{+2} to the extracellular DNA present in the matrix (Rose, 2000; Espinosa-Urgel et al., 2000; Hinsa et al., 2003; Das et al., 2014).

P. gingivalis strain W83 is encapsulated and has been showned to be more cytotoxic and likely to spread in the host (Lamont & Jenkison 1998; Nelson et al 2003). Capsule is showed to affect the surface properties of the cell, and thus affect cell-cell and cell-surface interactions (Davey & Duncan 2006; Lo et al., 2010). It has been shown that when the glycosyltransferase gene is knockout, once involved in the synthesis and transport of capsular polysaccharide, the adhesion is favored (Davey & Duncan 2006). Thus, the formation of *P. gingivalis* W83 biofilm in the presence of $CaCl_2$, would be an effect on the capsule with consequent favor of electrostatic bonds between the outer membrane and coated substrate.

Oral biofilm *in vitro* models have been a tool for the understanding of architecture and physiology of biofilms (Welch et al., 2012). These models allow studying factors of virulence and antimicrobial resistance (Lebeaux et al., 2013). Our study was the first to verify the effect of $CaCl_2$ on the formation of the *P. gingivalis* biofilm and to obtain a mature and robust biofilm with the W83 strain. Since biofilm formation is essential to the persistence of this organism, such methodology can contribute to study of the nutritional and environmental parameters that regulate development, as well identify the genes that are expressed during different stages of

biofilm growth and lead to a greater understanding of how to control this opportunistic oral pathogen (Donlan, 2001).

Conclusion

The finding data presented, concludes that $CaCl_2$ is an increase of the *in vitro* formation of the robust and viable *P. gingivalis* biofilm monospecies.

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CONCLUSÃO

Como base nos dados apresentados pela pesquisa, conclui-se que o $CaCl_2$ aumenta a formação *in vitro* do biofilme viável, mono-espécie, de *P. gingivalis.*

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