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PAULA VENTURA DA SILVEIRA

NOVAS TERAPIAS NO CONTROLE DE BIOFILMES ORAIS

Fortaleza
2017

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Odontologia. Área de concentração: Clínica Odontológica

Orientadora: Prof.(a). Dr.(a). Iriana Carla Junqueira Zanin dos Santos.
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RESUMO

Os biofilmes estão relacionados a maioria das doenças orais e os agentes antimicrobianos podem ser utilizados como tratamentos alternativos à remoção mecânica desses biofilmes. Diante da emergência de resistência microbiana frente ao uso de antimicrobianos convencionais, tem aumentado o interesse em terapias que tornem o surgimento de resistência improvável. Nesse contexto, a terapia fotodinâmica antimicrobiana e o uso dos plasmas de baixa temperatura, ambos baseados na formação de espécies reativas de oxigênio, podem ser utilizados. Assim, o objetivo deste estudo foi analisar o uso de novas terapias no controle dos biofilmes orais. No primeiro capítulo foi realizado um experimento com a fototerapia sobre a formação de biofilme de *Candida albicans* (SN 425). O biofilme foi exposto a luz azul e vermelha (400-690 nm), duas vezes ao dia. Foram utilizados a Clorexidina 0,12% e o NaCl (0,89%) como grupos controle. As amostras desses biofilmes foram analisadas através da contagem das unidades formadoras de colônia (UFC), peso seco e polissacarídeos. A análise de peso seco dos biofilmes de *C. albicans* revelou uma redução significativa após o tratamento com luz vermelha e luz azul quando comparados aos grupos controle. Embora essa redução não tenha sido acompanhada pela redução na viabilidade das células, concluiu-se que o tratamento duas vezes por dia com luz azul e vermelha foi um mecanismo promissor para a inibição do desenvolvimento de biofilmes *C. albicans*. No segundo capítulo, foi realizado um experimento com o plasma de baixa temperatura com oito voluntários que utilizaram dispositivos intra-orais contendo blocos de esmalte bovino, tratados 10 vezes ao dia com uma solução de sacarose a 10% durante 7 dias. Decorrido o período experimental, os blocos de esmalte receberam tratamento durante 5 minutos de plasma, clorexidina 0,12%, gás argônio ou solução salina 0,89%. As amostras de biofilmes foram recolhidas e processadas para a detecção molecular das bactérias de interesse. Os resultados demonstraram que o tratamento dos biofilmes com plasma reduziu significativamente as concentrações de *Streptococcus mutans*, *Lactobacillus acidophillus*, *Streptococcus mitis*, *Actinoomyces naeslundis* e *Bifidobacterium* ($p < 0,05$) em comparação com outros grupos de tratamento. As concentrações de *Lactobacillus casei* e *Streptococcus gordonii* não foram influenciados pelo tratamento com plasma de baixa temperatura. Este estudo forneceu resultados para uma melhor compreensão entre as diferenças nas microbiotas após diferentes tratamentos antimicrobianos sobre os biofilmes. Em conclusão, o uso dos plasmas de baixa temperatura e da fototerapia são terapias efetivas na inativação e/ou no desenvolvimento de biofilmes orais patogênicos associados a diversas doenças que atingem a cavidade bucal dos seres humanos.

Palavras-chaves: Biofilme dentário, Gases em Plasma, biologia molecular, fototerapia.

ABSTRACT

Biofilms are related to most diseases and antimicrobial agents can be used as alternative treatments to mechanical removal of biofilms. Faced with the emergence of microbial resistance versus the use of conventional antimicrobials, interest in therapies that make the emergence of resistance unlikely has increased. In this context, antimicrobial photodynamic therapy and the use of low temperature plasmas, both based on the formation of reactive oxygen species, can be used. Thus, the objective of this study was to analyze the use of new therapies in the control of oral biofilms. In Chapter 1, an experiment was carried out with a phototherapy on the biofilm formation of Candida albicans (SN 425). The biofilm was exposed to blue or red light (400-690 nm), without the use of photosensitizers, twice a day. Chlorhexidine 0.12% and NaCl (0.89%) were used as control groups. Biofilm samples were analyzed by counting the colony forming units (CFU), dry weight and polysaccharides. A dry weight analysis of the biofilm product of *C. albicans* revealed a significant reduction after treatment with blue and red light when compared to the control groups. Although it has not been accompanied by the reduction in viability of the cells and considering the growth of this microorganism in the form of hyphae, it was concluded that the treatment twice a day with blue and red light it is a promise therapy for inhibition of *C. albicans* biofilms. In the second chapter, a low-temperature plasma experiment was performed with volunteers using intraoral devices containing blocks of bovine enamel, treated 10 times daily with a solution of 10% sucrose for 7 days. After the experimental period, the enamel blocks were treated for 5 minutes of plasma, Chlorhexidine 0.12%, argon gas or 0.89% saline solution. The biofilm samples were collected and processed for identification of the genes of the bacteria of interest. The results demonstrated that treatment of plasma biofilms significantly reduced concentrations of *Streptococcus mutans*, *Lactobacillus acidophillus*, *Streptococcus mitis*, *Actinomyces naeslundi and Bifidobacterium* group ($p < 0.05$) compared to other treatment groups. As concentrations of *Lactobacillus casei* and *Streptococcus gordonii* were not influenced by treatment with low temperature plasma. This study provides results for a better understanding between the difference of microbiotes after different antimicrobial treatments in biofilms. In conclusion, the use of low temperature plasmas and photodynamic therapy are effective therapies in inactivation of bacteria and/or in the development inhibition of pathogenic oral biofilms associated with various diseases that generate the oral cavity of humans.

Keywords: Dental plaque, plasma gases, molecular biology, phototherapy

SUMÁRIO

1 INTRODUÇÃO GERAL

A grande maioria dos microrganismos na natureza é encontrado ligada às superfícies, onde crescem e formam biofilmes. Os biofilmes consistem em uma ou mais comunidades de microrganismos, embebidos em uma matriz, aderidos uns aos outros e/ou a superfícies ou interfaces (COSTERTON *et al*., 2005, FLEMMING *et al*., 2016). O biofilme oral compreende estruturas tridimensionais complexas, formado por comunidades de multiespécies microbianas sobre o tecido oral (HE, *et al*., 2015; HOJO, *et al*., 2009), incorporados em uma matriz de polissacarídeo extracelular (REESE; GUGGENHEIM B., 2007).

Geralmente, a dinâmica de formação de um biofilme ocorre em etapas distintas. Inicialmente temos a adesão dos organismos denominados colonizadores primários, que se aderem à superfície, comumente contendo proteínas ou outros compostos orgânicos. As células aderidas passam a se desenvolver, originando microcolônias que sintetizam uma matriz exopolissacarídica, que passa a atuar como substrato para a aderência de microrganismos denominados colonizadores secundários. Estes colonizadores secundários podem se aderir diretamente aos primários, ou promoverem a formação de coagregados com outros microrganismos (RICKARD *et al*., 2013). Assim, o biofilme corresponde a uma "entidade" dinâmica, pois, de acordo com os microrganismos que o compõem, teremos condições físicas, químicas e biológicas distintas. Estas alterações fazem com que cada biofilme seja único, de acordo com os microrganismos presentes. Nesse sentido, ao longo do tempo a composição microbiana dos biofilmes geralmente sofre alterações significativas (JENKINSON; LAPPIN-SCOTT, 2011).

Um biofilme natural formado sobre a superfície dos dentes pela aderência de diferentes espécies de bactérias e de matriz extracelular com glucanos solúveis e insolúveis é chamada de placa dentária. Ela é afetada por diversos fatores externos, como a dieta, a composição da saliva e o fluxo salivar (MARSH, 2015). A placa dentária é o biofilme mais extensivamente pesquisado (KOLENBRANDER, 2010).

Mais de 700 espécies de bactérias foram identificadas em amostras de biofilme oral (BLANC *et al*, 2014; KUTSCH, 2014), a composição de espécies bacterianas varia entre os indivíduos, os locais na cavidade oral, a dieta e o comportamento (BLANC *et al*., 2014; KUTSCH, 2014). Entre estes microrganismos, as espécies estreptococos compreendem a maioria da população (PRATTEN *et al*., 2013) que juntamente com Bacilos Gram Positivos são as primeiras bactérias a colonizar a película sobre a superfície do dente (MASH, 2014).

Os *Streptococcus sanguinis, Streptococcus oralis*, *Streptococcus gordonii, Streptococcus mitis, Actinomyces naeslundii, Fusobacterium nucleatum*, *Capnocytophaga ochraceae*, *Streptococcus mutans* e *Streptococcus sobrinus* podem estabelecer quando existir disponibilidade frequente de sacarose, portanto, em função da dieta do hospedeiro (ROSANA; LAMONTB, 2000; ZIJNGE *et al*., 2010; RICKARD *et al*., 2013).

O biofilme é composto por bactérias Gram-positivas e negativas, sofrendo interferências do fluxo salivar e dos componentes de defesa do hospedeiro, tornando-se patogênico para os tecidos periodontais e dentais em decorrência da susceptibilidade do hospedeiro. O biofilme pode ser encontrado na região supragengival ou subgengival. O biofilme supragengival caracteriza-se pela presença qualitativa de microrganismos Gram-positivos que servem de substrato para a formação do biofilme subgengival em decorrência da sua permanência sobre a estrutura dental. *S. mutans* encontra-se no complexo microbiano relacionado ao biofilme supragengival enquanto *Porphyromonas gingivalis* relacionado ao biofilme subgengival (CREA, 2014).

As bactérias associadas ao biofilme oral resistem dentro do biofilme supragengival, composto basicamente com bactérias aeróbias sacarolíticas (SOCRANSKY *et al*, 1998). A natureza dos biofilmes subgengivais é mais complexa compreendendo bactérias anaeróbias e proteolíticas tais como *Porphyromonas gingivalis, Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, além de, *Staphylococci* (SOUTO *et al*, 2006).

As espécies de *Cândida albicans* também são muito prevalentes, sendo um fungo normalmente encontrado no corpo humano sem, contudo, ocasionar um processo patológico em indivíduos saudáveis. Estes microrganismos podem facilmente ser recuperados da mucosa oral, trato gastrointestinal, vagina e pele em condições de saúde. Entretanto, sob certas circunstâncias, este microrganismo pode causar uma infecção, conhecida como candidose, que acomete principalmente pacientes imunodeficientes, podendo evoluir para infecção sistêmica (SENEVIRATNE *et al*, 2008).

A incidência de infecções hospitalares por fungos tem aumentado expressivamente nas ultimas décadas. Até 60% dos óbitos oriundos de infecções hospitalares são causadas por fungos (TAMURA, 2007). Neste contexto, as espécies do gênero *Cândida* tem sido os agentes mais frequentemente encontrados, correspondendo a cerca de 80% das infecções fúngicas de origem hospitalar e são a quarta causa de infecção da corrente sanguínea, conduzindo ao óbito em torno de 25 a 38% dos pacientes que desenvolvem candidemia (EGGIMANN, 2015). O biofilme forma uma matriz complexa e, dada a extraordinária resistência dos organismos dentro do biofilme aos mecanismos de defesa do

hospedeiro, o primeiro passo para o controle destes microrganismos seria a remoção mecânica do biofilme (SOCRANSKY *et al*, 2002).

As doenças orais relacionadas com biofilmes afetam a maioria da população mundial. Uma descoberta importante de relevância clínica em relação aos microrganismos que crescem sobre uma superfície é a sua maior resistência aos agentes antimicrobianos (CERI *et al*., 1999; GILBERT *et al*., 2012). Desde 1970 tem sido verificado um aumento significativo nessa resistência, resultante da utilização, algumas vezes indiscriminada, de agentes antimicrobianos (BRUNTON *et al*, 2010). Segundo Tobudic *et al* (2010) os microrganismos dos biofilmes podem ser de 10 a 1000 vezes mais resistentes aos antibióticos do que as bactérias geneticamente idênticas, isso ocorre devido a própria resistência mediada pela célula, como as mutações naturais evolutivas das bactérias e a transferência de genes de resistência homólogos (HOIBY *et al*, 2011). Ainda, segundo Diaz (2015), através da resistência mediada pela presença do biofilme que resulta na deficiente penetração e na barreira de difusão dos antibióticos na matriz polissacarídica estável, através do crescimento lento e resposta geral ao stress, da heterogenicidade e do *quorum sensing*, que consite em um sistema de comunicação entre as bactérias, no qual sintetizam compostos sinalizadores de baixo peso molecular, os autoindutores bacterianos, que são excretados para o meio ambiente, as bactérias detectam a presença dos mesmos e respondem ativando ou reprimindo certos genes (MAH e O'TOOLE, 2001 e VIANA, 2016).

Além disso, as células crescidas como biofilme expressam propriedades distintas das células planctônicas, uma das quais é uma resistência aumentada aos agentes antimicrobianos. Trabalhos indicam que a estrutura física e/ou química dos exopolissacarídeos ou outros aspectos da arquitetura do biofilme também podem conferir resistência da comunidade bacteriana. Os microrganismos cultivados em biofilme podem desenvolver um fenótipo resistente a biocidas específico. Devido à natureza heterogênea do biofilme, é provável que existam múltiplos mecanismos de resistência atuando em uma única comunidade. Pesquisas esclarecem sobre como e por que as comunidades microbianas ligadas à superfície desenvolvem resistência aos agentes antimicrobianos (MAH e TOOLE, 2001).

Fatores que adicionalmente influenciam nessa resistência seriam a idade e a estrutura do biofilme, que podem restringir a penetração do agente antimicrobiano e a modificação do fenótipo, diferindo do seu estado planctônico, após formarem uma matriz de polissacarídeo extracelular, o que os tornam mais tolerantes aos antibióticos e às forças de atrito (MARSH, 2004).

Diante disso, nenhum dos agentes antiplaca disponíveis atualmente no mercado é

totalmente eficaz contra biofilmes maduros, tornando necessário a adoção de métodos alternativos para o tratamento da placa dentária sem prejudicar os tecidos saudáveis, bem como para o tratamento das doenças bucais (WILLIANS, 2011).

Os métodos alternativos desenvolvidos para erradicar bactérias indesejáveis incluem a terapia fotodinâmica antimicrobiana (TFA), fototerapia, antimicrobianos nanoparticulados, peptideos antibacterianos e de Plasma de Baixa Temperatura (PBT) (LINS *et al*., 2015; BROGDEN; BROGDEN, 2011; CARJA *et al*., 2009; HASAN; CRAWFORD; IVANOVA, 2013; REN *et al*., 2009).

Nesse contexto, a fototerapia e o uso do plasma de baixa temperatura, ambos baseados na formação de espécies reativas de oxigênio, podem ser utilizados. A Terapia fotodinâmica antimicrobiana é uma fototerapia baseada na utilização de substâncias de origem endógena ou exógenas que são ativadas na presença da luz gerando espécies reativas de oxigênio (ROS) podendo sensibilizar sistemas biológicos (DONNELY, 2008).

A utilização dessa terapia é conhecida em diversas áreas e de modo geral, atua promovendo uma desinfecção local pela associação de luzes com fotossenssibilizador (FS) endógeno ou exógeno de comprimento de onda complementar. A terapia fotodinâmica foi inicialmente idealizada para o tratamento do câncer e sua ação antimicrobiana só começou a ser efetivamente estudada nas últimas duas décadas, quando no casoda odontologia, começou a ser testada tendo como alvo as células bacterianas envolvidas no desenvolvimento das doenças bucais (FEUERSTEIN, 2015).

A terapia fotodinâmica antimicrobiana pode ser indicada como alternativa adicional à terapia antimicrobiana convencional para matar bactérias orais (FEUERSTEIN, 2015; XIA; KOO, 2010; HAMBLIN, 2004; ROMLING, 2012). Baseia-se no uso de fotosensibilizadores extrínsecos, moléculas absorventes de luz que iniciam uma reação fotoquímica quando expostas à luz de um comprimento de onda específico. Este processo de fotoquímica leva a formação de espécies de oxigênio reativo (ROS), o que pode causar danos irreversíveis aos compostos essenciais de células bacterianas e alterar o metabolismo celular, resultando em morte bacteriana (DOUGHERT, 1998). A maior limitação do TFD é a dificuldade de penetração do corante através das profundidades do biofilme, de modo que a fototerapia sem a utilização do corante pode ser uma alternativa a utilização da TFD. Assim, fototerapia com luz azul e vermelha (400-690 nm de comprimento de onda) parece ser uma alternativa promissora para o TFD, uma vez que excede esse desafio. Seu mecanismo antimicrobiano é semelhante ao TFD, no entanto, a morte bacteriana parece envolver a ativação de fotosendibilizadores endógenos em bactérias, como flavinas e citocromos, o que

pode levar à produção de ROS (FEUERSTEIN, 2015).

Outra terapia emergente no controle das infecções orais e baseada na produção de espécies reativas de oxigênio é o uso do Plasma de Baixa Temperatura, uma recente tecnologia para o tratamento antimicrobiano, sendo uma alternativa de grande potencial aos tratamentos tradicionais, tais como antibióticos e atua também como um promotor de cicatrização de feridas, tornando-se uma ferramenta promissora em uma variedade de aplicações biomédicas, com particular importância ao combate de infecções (MAI-PROCHNOWA *et al*., 2014). O Plasma é o quarto estado da matéria, é um gás ionizado neutro e pode ser gerado utilizando uma variedade de gases ou misturas de gases como: argônio, hélio, ozônio ou gás oxigênio. É constituído por partículas em interação permanente, como fótons, elétrons, íons positivos e negativos, átomos, radicais livres e moléculas excitadas e não excitadas (MOREAU; ORANGE; FEUILLOLEY, 2008), as quais podem contribuir para as suas propriedades antibacterianas.

Em geral, existem dois tipos de plasma gasoso, que são classificados de acordo com as condições em que são criados, os plasmas térmicos são obtidos a alta pressão (≥105 Pa) e precisam de um maior poder substancial (até 50 MW), a temperatura do gás é quase a mesma para todos os componentes do plasma e pode ser muito elevada (5 a 20 × 103 K). Os plasmas não térmicos ou de baixa temperatura são obtidos a pressões mais baixas e utilizam um menor poder substancial. Estes últimos são caracterizados por uma temperatura muito elevada dos elétrons mais do que a do gás (temperatura macroscópica) e consequentemente não apresentam um equilíbrio termodinâmico local (MAI-PROCHNOWA *et al*., 2014; SCHOLTZ *et al*., 2015). Pesquisas têm demonstrado que o tratamento com o Plasma à Baixa Temperatura (PBT) pode inibir completamente a formação de um biofilme rico em matriz (DUARTE *et al*., 2011). O Plasma surge como um tratamento físico eficaz com efeito antimicrobiano, para bactérias, parasitas, fungos, esporos e vírus (WU, 2013).

As vantagens do PBT e da fototerapia, sobre terapias antibióticas são que eles podem ser utilizados para um tratamento localizado, fornecendo uma resposta bactericida rápida, o que torna a probabilidade do desenvolvimento de resistência bacteriana improvável, e essas terapias demonstram efeitos colaterais mínimos. Além disso, a temperatura é compatível com os tecidos mamíferos, o que incentiva a utilização *in vivo* (FLUHR *et al*., 2012; PATTERCKE *et al*., 2012). Assim, essas tecnologias têm recebido crescente atenção como possíveis terapias para o tratamento e/ou prevenção de biofilmes.

Até esta data, tem havido algumas investigações em relação ao tratamento de bactérias orais com PBT e com a fototerapia antimicrobiana (BIN *et al*., 2016). Ao contrário

dos efeitos antimicrobianos, ainda são incipientes dados sobre como o PBT afeta a estrutura e a formação do biofilme e da expressão gênica do mesmo. Da mesma forma, ainda há carência de informações de como essas novas terapias antimicrobianas atuam em biofilmes orais

2 PROPOSIÇÃO

GERAL

Analisar o uso de novas terapias aplicadas ao controle do biofilme oral.

ESPECÍFICOS

- Descrever os efeitos da fototerapia com a luz azul ou vermelha no desenvolvimento do biofilme de *C. albicans*.
- Avaliar o efeito antimicrobiano do plasma de baixa temperatura sobre biofilmes orais formados *in situ*, através da identificação de parte da população microbiana.

3 CAPÍTULOS

REGIMENTO INTERNO

Esta tese está baseada no Artigo 46 do Registro Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertação de mestrado e tese de doutorado e permite a inserção de artigos científicos de autoria e co-autoria do candidato. Por se tratar de pesquisa envolvendo seres humanos, ou parte deles, o projeto de pesquisa deste trabalho foi submetido à apreciação do Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade Federal do Ceará via Plataforma Brasil, tendo sido aprovado sob Caae - 40975514.0.0000.5054 (ANEXO A). Assim sendo, essa tese de doutorado é composta por dois capitulos que contém dois artigos que serão submetidos para publicação nos periódicos "Laser in Medical Science" (ANEXO B) and "Clinical Oral Investigation" (ANEXO C).

Capítulo 1: Effect of twice-daily red and blue light treatment on *Candida albicans* biofilm development

"Laser in Medical Science" – qualis capes A2

Capítulo 2: Antimicrobial effect of Low Temperature Plasma on oral biofilm formed *in situ*: molecular partial identification of microbial population

"Clinical Oral Investigation" – qualis capes A1

3.1 Capítulo 1:

Effect of twice-daily red and blue light treatment on *Candida albicans* biofilm development

Paula Ventura da Silveira, Beatriz Helena Dias Panariello, Cecília Atem Goncalves de Araújo Costa, Shawn Maule, Shane Maule, Malvin Janal, Iriana Carla Junqueira Zanin, Simone Duarte

Abstract

Introduction: The use of blue light or red light has been proposed as a direct means of affecting local bacterial infections; however, the use of light to prevent the biofilm development of *Candida albicans* has received less attention. The aim of this study was to determine how the treatment with red and blue lights affects the development and composition of a matrix-rich *Candida* biofilm.

Methods: Red and blue light treatment were applied to *Candida albicans* (SN 425) biofilms twice-daily for 48 h**.** All the experiments were repeated on five separate occasion with two replicaties. After 18 h biofilm formation, the biofilm was exposed to non-coherent blue light and red light (Luma- Care; 420 nm and 635 nm). The distance between the light source tip and the exposed sample was 1.0 cm and the parameters adopted were energy density of 72 J cm^{-2} and time exposure of 12. 56 min for blue light and 18 J cm^{-2} for 27.3 s, 35 J cm^{-2} for 1 min, and 53 J cm^{-2} for 2 min for red light. Positive and negative control groups were treated twice-daily with 0.12% chlorhexidine (CHX) (1 min) and 0.89% NaCl (1 min), respectively. Biofilms were analyzed for colony forming units (CFU), dry-weight, and exopolysaccharides (EPS-insoluble and EPS-soluble).

Results: *C. albicans* biofilms dry-weight were significantly reduced by the treatment with red and blue light. The EPS-soluble content was mainly reduced by twice-daily exposure to blue light while EPS-insoluble exhibited major reduction via twice-daily treatment with red light for 1 min.

Conclusion: Twice-daily treatment with blue and red light is a promising mechanism for the reduction of matrix-rich *C. albicans* biofilm development.

Keywords: *Candida albicans***,** Biofilm, Red Light, Blue light, Phototherapy.

Introduction

Candida albicans is the main specie associated with oral candidiasis and has been increasingly observed in both in immune-compromised and non-compromised individuals [1]. In recent years, there has been a significant increase in the incidence of oral candidiasis. Several factors are thought to be responsible for this increase, including a growing incidence of diabetes, increased age life expectancies, the growth in HIV-infection and the AIDS epidemic, a widespread use of immunosuppressive therapy, the use of broad spectrum antibiotics and invasive clinical procedures such as solid organ or bone marrow transplantation. In this context, *C. albicans* is a frequent fungal biofilm-forming pathogen that can cause life-threatening infections by colonizing medical and dental devices (i.e. prostheses, implants and catheters) [2]. Dimorphism is an important characteristic experienced by *C. albicans* in response to adverse environmental conditions, which increases its virulence. *C. albicans* can undergo from around yeast cells to long filamentous cells named hyphae [3]. The yeast form facilitates the colonization of different sites while the hyphal form has an important role in causing disease by invading epithelial cells and causing tissue damage [3].

C. albicans attachment to mucosal tissues and to abiotic surfaces and the formation of biofilms are crucial steps for its survival and proliferation in the oral cavity[4]. It is estimated that most microorganisms in nature occur in biofilms [5]. Biofilm growth starts when planktonic cells adhere to a surface and the proliferation of the yeast cells across the substrate surface starts, as well as the beginning of hyphal development. The final step of biofilm development is the maturation stage, in which yeast-like growth is repressed, hyphal growth is augmented, and extracellular matrix encases the biofilm [6]. Exopolysaccharides (EPS), proteins, lipids, nucleic acids, lipoteichoic acids (LTA), and even lipopolysaccharides have been identified in the matrices of bacterial biofilms [6]. The analyses of EPS matrix formation could advance the current understanding of the development process and structural organization of oral biofilms, which would be essential for designing novel and effective antibiofilm therapies. Furthermore, biofilm formation is one of the most important attributes for virulence in *C. albicans* species and contributes to increased resistance to the current antifungal agents, environmental stress and host immune mechanisms [7]. It has been shown that *C. albicans* polysaccharides in the extracellular matrix are composed by β-1,6 glucan (EPS-insoluble) and α-mannan (EPS-soluble) that interact to form a mannan-glucan

complex (MGCx) [8;9]. This exopolysaccharide interaction of *C. albicans* is essential for protection of the biofilm from drug treatment [10].

The photodynamic antimicrobial chemotherapy (PACT) has been indicated as an alternative to conventional antimicrobial therapy to kill oral bacteria. It is based on the use of extrinsic photosensitizers, light-absorbing molecules that initiate a photochemical reaction when exposed to light of a specific wavelength [11]. A previous study investigated the antimicrobial effect of blue (wavelength range, 400–440 nm) and red (wavelength range, 570–690 nm) light-emitting diode (LED) into different exposure times to active different concentrations of curcumin and toluidine blue on planktonic suspensions of *Streptococcus mutans*. It was observed that the LED device in combination with curcumin and toluidine blue promoted an effective photoinactivation of *S. mutans* suspensions at ultrashort light illumination times. However, the greatest PACT limitation is the challenge for the photosensitizer to penetrate through the depths of the biofilm[35]

The phototherapy seems to be a promising alternative for PACT since it exceeds this challenge. Its antimicrobial mechanism is like PACT, however, the bacterial killing seems to involve the activation of endogenous photosensitizers in bacteria, such as flavins and cytochromes, that may lead to production of ROS (Reactive Oxygen Species). The antimicrobial effect of blue light alone has been demonstrated in *S. mutans* biofilms[12]; however, the effect of blue and red light in *C. albicans* biofilms has never been investigated. Therefore, the aim of this study was to determine how the treatment with blue light and red light affects the development and composition of a matrix-rich *Candida* biofilm.

Methods

Light Sources

A noncoherent light was used (LumaCare LC-122 A, LumaCare Medical Group, Newport Beach, CA, USA). This device offers interchangeable flat probes (beam diameter=12 mm; spot area=113.1 mm^2) at specific frequencies that are connected with a simple interlocking connection. To this study, two different fiber optic probes were used to cover the blue and red-light spectrum. The blue light (wavelength range, 400–440 nm) with a central wavelength peak at 420±20 nm at power density of 95.5 mW cm^{-2} (set power=105 mW). The radiant exposures tested to this specific wavelength was

72 J.cm^{-2}. Thus, the biofilms were irradiated for 12.5 min. The red light (wavelength range, 570–690 nm) provided an absorption spectrum with a central wavelength at 635±10 nm with a power density of 1,460 mW cm^{-2} (set power =1,650 mW) and 18, 35, and 53 J cm^{-2} of radiant exposures. The exposure time corresponding to each tested radiant were 2 min, 1 min and 27.3 s, respectively. A work distance of 5 mm between the light source and biofilms surface was applied as it is a safe distance to avoid heating sample[36].

Inoculum

The biofilm was obtained from the strain *C. albicans* SN 425. The microorganism stored at -80ºC were seeded onto Petri dishes with SDA (Sabourand dextrose agar) culture and incubated at 37°C for 48h. Then, around 5 colonies of the microorganism were taken with a loop and added in YNB medium (Yeast Nitrogen Base- DIFCO, Detroit, Michigan, USA) supplemented with 100 mM of glucose. The pre-inoculum was incubated at 37°C. After 16h of incubation, the pre-inoculum was diluted with fresh YNB medium supplemented with 100 mM glucose (1:10 dilution). These inoculum cultures were incubated at 37°C until the strain reached the mid-log growth phase (8 hours, $OD_{540nm} \tilde{=} 0.400$ nm). Then, the inoculums were adjusted to reach 10^7 cells/mL.

Biofilm formation and phototherapy

One milliliter of the inoculum of each strain was added to the wells of a 24-well polystyrene plate. The culture plate was incubated at 37°C for 90 min (adhesion phase). After this period, the wells were washed twice with sterile 0.89% NaCl solution to remove non-adhered cells. Afterwards, one (1) mL of RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, Missouri, USA) at pH 7 was added to each well. After 18 hours of initial biofilm formation, the biofilm was exposed to red and blue light twice daily (9 am and 3pm), until 48 hours of biofilm formation. Positive and negative control groups were treated twice-daily with 0.12% chlorhexidine-CHX (1 min) and 0.89% NaCl (1 min), respectively.

Biofilm analysis

At the end of the experimental period, the biofilms were washed twice with 1 mL of sterile 0.89% NaCl solution. For biofilm removal, 2 mL of sterile 0.89% NaCl

solution were added to the plates and the wells surfaces were gently scraped with a sterile spatula. The removed biofilms were added to sterile tubes. The tubes containing the removed biofilms were vortexed and an aliquot of 100 ml was separated for the dry-weight[13] and another aliquot of 100 µl was separated for colony forming units (cfu/mL). The remaining content was centrifuged (10000 rpm, 10 min). The supernatant had 1 mL collected for EPS-soluble analysis by phenol: chloroform method [14] and the biofilm pellet was resuspended and washed with milli-Q water; this procedure was repeated three times. Then, the biofilm pellet was resuspended with 1 mL of milli-Q water and this aliquot was used for the EPS-insoluble analysis by phenol: chloroform method [14].

Statistical analyses

All the experiments were repeated on five separate occasion with two replicates. The polysaccharide content was normalized by the dry-weight. Colony forming units was transformed to Log_{10}. Data was analyzed by two-way ANOVA and Tukey's test. The *p* value was <0.05 for statistical significance.

Results

Figure 1 shows the results of Log_{10} CFU/mL of *C. albicans*. The First bar represents the negative control. Each group was compared with the negative and positive control. There was significant difference in Log_{10} CFU/ml between the negative and positive control samples ($p<0.05$). However, there was no significant difference between light groups and negative control ($p>0.05$).

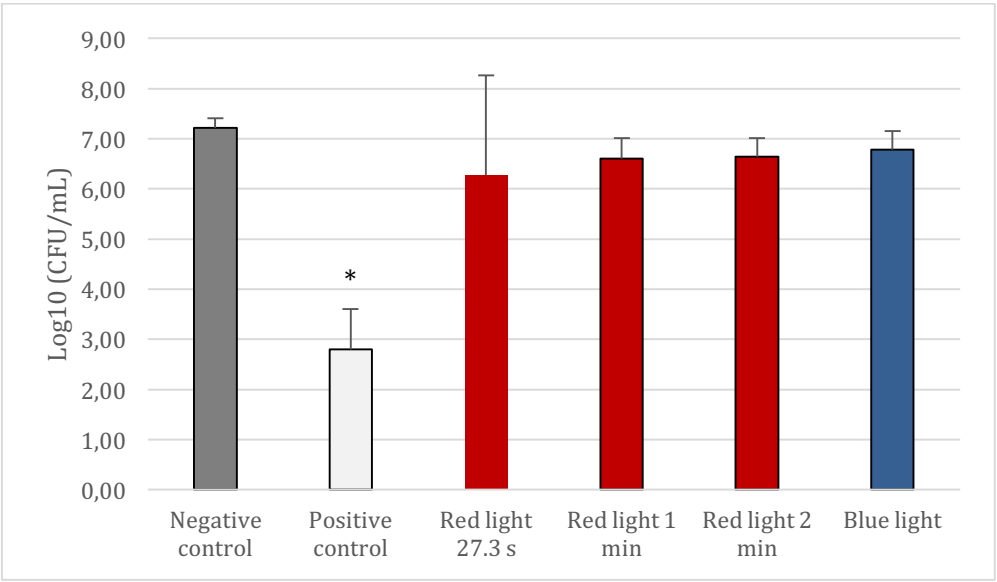

Fig 1. Mean and standard deviations of Log_{10} CFU/mL of *C. albicans*. Comparison was made between the twice-daily light treatment and the controls-0.12% CHX (positive control) and 0.89% NaCl (negative control). The * points to significant differences (p<0.05) in comparison to the other groups.

The figure 2 shows the results of the dry-weight (mg) of *C. albicans* biofilms after periodic light treatment. A significant reduction of the dry-weight in all light-treated samples in comparison to the negative control was observed and the reduction values observed in the light-treated groups was statistically similar to the positive control.

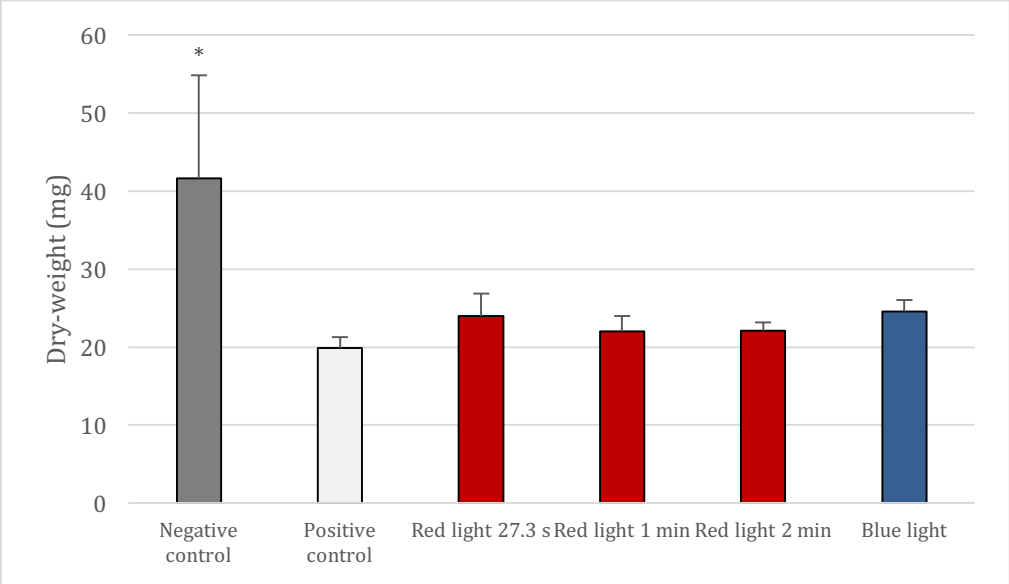

Fig 2. Mean and standard deviations of dry-Weight (mg) of *C. albicans* biofilm after the twice-daily treatment with red and blue light and with 0.12% CHX (positive control) and treatment with 0.89% NaCl (negative control). The * points to significant differences (p<0.05) in comparison to the other groups.

Figure 3 shows the results of *C. albicans* EPS-soluble and -insoluble contents after the twice-daily treatment with red and blue light and with 0.12% CHX (positive control) and treatment with 0.89% NaCl (negative control). It was observed that twice-daily light exposure to red light for 1 and 2 min and to blue light for 12 min 56 s numerically reduced the EPS- soluble in comparison to the negative control, mainly the blue light treatment. On the other hand, the EPS-insoluble content was numerically reduced by twice-daily exposure to red light for 1 min in comparison to the negative control, to the blue light and to the other periods of exposure to red light. Thus, there is a tendency of EPS-soluble reduction by twice-daily treatment with the blue light and a tendency of reduction of EPS-insoluble by twice-daily treatment with red light for 1

min. Further studies with the combination of the blue light for 12 min 56 s with the red light for 1 min for reduction of matrix-EPS in *C. albicans* infections could be performed.

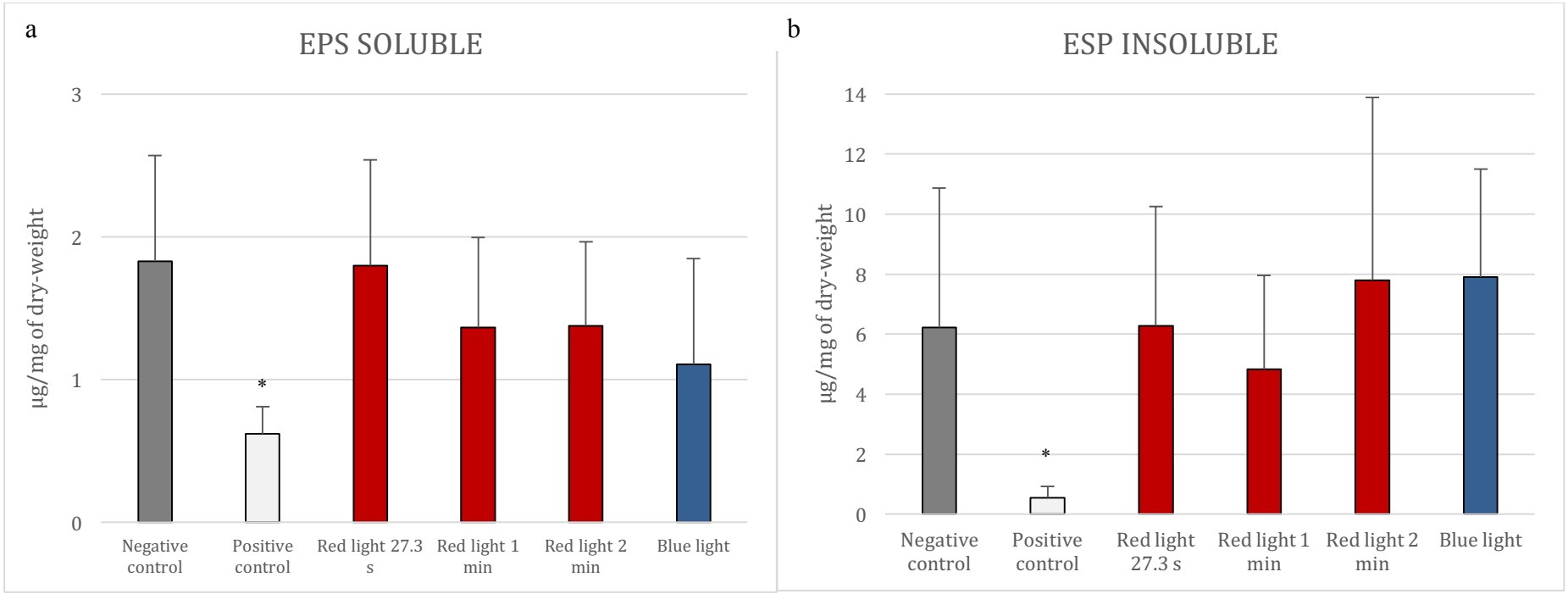

Fig 3. Mean values and standard deviations of EPS-soluble and insoluble content in *C. albicans* biofilm (µg/mg of dry-weight) after twice-daily light treatment compared to twice-daily treatment with 0.12% Chlorhexidine (positive control) and twice-daily treatment with 0.89% NaCl (negative control). The * points to significant differences (p<0.05) in comparison to the other groups.

Discussion

C. albicans is the most frequent specie isolated from superficial and systemic fungal infections and is associated with high rates of mortality[37]. There is an increasing number of strains of this microorganism that are resistant to antifungal agents[38]. Treatments of oral infections caused by *Candida* use topical antifungal medication, such as Nystatin[15]; and systemic antifungal medication, such as Fluconazole[16]. Due to the antifungal resistance and difficulties associated with the use of conventional medications, antimicrobial photodynamic chemotherapy (PACT) has been indicated for inactivating *Candida* and for the treatment of superficial fungal infections[17;18;19]. Studies have demonstrated that species of *Candida* present susceptibility to PACT [18]. However, this method has limitations, such as non-selective antimicrobial characteristics and difficulty to penetrate to the depths of the biofilm, resulting in less effectiveness in biofilms[39]. As an alternative to PACT, a previous study described the use of blue light to prevent the biofilm development of *Streptoccocus mutans* in vitro[12]. The study found that that twice-daily treatment prevented in vitro *S. mutans* biofilm matrix development, being more effective in reducing the production of EPS-insoluble than the 'gold-standard' anti-plaque 0.12% chlorhexidine [12]. However, the effect of blue and red light in *C. albicans* biofilms has never been investigated. Therefore, the aim of this study was to determine how the treatment with blue light or red light affects the development and composition of a matrix-rich *Candida* biofilm.

The light is an essential environmental cue for various organisms [20]. Light is the major source of energy in the biosphere, and an essential signal that controls growth, development, and behavior of many different physiological mechanisms in most organisms. Long term experience with phototherapy for the treatment of jaundice, cancer and dermatological conditions has demonstrated its safety as well as its effectiveness [24]. LumaCare™ device is a source of light that produces the whole spectrum of visible light by changing different probes at specific wavelenghts [37]. The light source presents a high potency (1,460 mW cm^{-2}) in the red wavelength, thus, the exposure of the biofilms to this light was short (2 min, 1 min and 27.3 s) [37]. On the other hand, the blue LED (95.5 mW cm−2) has a lower penetration depth into the tissue compared to red light due to its low potency associated with scattering and absorption by biomolecules, resulting in longer illumination time (12.56 min) [37].

Exopolysaccharides (EPS) are abundant polymers in the biofilm matrix of *C. albicans* [21;8]. The *Candida* resistance to medications is multifactorial, being related to the physiological state of the cells, to the activation of drug efflux pumps and to the protective effect of the EPS of the extracellular matrix [33]. β-glucans from the extracellular matrix of *C. albicans* biofilms (EPS-insoluble) bind to fluconazole [22] and amphotericin B, preventing the diffusion of these drugs into the biofilm[23]. Because the ECM of *C. albicans* is composed by a complex interaction between

soluble (α-mannan) and insoluble (β-1,6 glucan) EPS, the present study focused of on these components. Results on the photodynamic degradation of individual components of *Candida* biofilm matrix are scarce.

The present study observed a significant reduction of the total biomasses (dry-weight) of the biofilms after the treatments with red and blue lights (Figure 2). The total biomass is defined as the total weight of the biofilm after the treatments and the washes of the biofilm. By reducing the biomasses, the lights prevented biofilm development. On the other hand, the CFU count was not affect. Thus, the reduction of the biomasses happened because of the reduction of the matrix components. We observed a numerical reduction of EPS-soluble that might have influenced in the significant reduction of the biomasses. However, *Candida* matrix have other important components, such as eDNA and proteins[40] that might have been affected by the photherapy but were not evaluated in this study. Thus, the limitation of our study is that we only evaluated EPS-soluble and -insoluble from the matrix of *C. albicans*. Further studies should evaluate the effect of red and blue light in eDNA and proteins.

In the present study, the twice-daily treatment with blue light resulted in a numerical reduction in the EPS-soluble content of *C. albicans* biofilms (Figure 3a). Moreover, the EPS-soluble was numerically reduced by the application of red light for 1 min twice a day (Figure 3a). The decrease in the concentration of polysaccharides with the twice-daily treatment with blue light and red light for 1 min indicates that the light interactions with matrix components may affect the cohesiveness and stability of the EPS and the leakage of polysaccharides from the biofilm, without causing a significant effect of cell viability (Figure 1). The reduction of polysaccharides from the matrix of *C. albicans* biofilms is important since these components are related to the protection of the biofilm from antifungals[8]. Thus, the twice-daily treatment of oral candidiasis with blue light for 12 min 56 and with red light for 1 min might function as an adjuvant to topic antifungal application, such as Nystatin.

Our results demonstrated the effect of blue and red light on *C. albicans* biofilms. To our knowledge, this is the first report of twice-daily use of blue and red light to prevent *C. albicans* biofilm development. This exposure prevented *C. albicans* biofilm development by reducing the biomasses. Moreover, even though no statistically differences for EPS reduction were noted, there is a high tendency of reduction of EPS-soluble by blue light for 12.56 min and red light for 1 min. There was no cfu/mL reduction by the treatments, so the reduction of the biomasses occurred by the reduction of matrix components. Thus, future studies with red and blue light therapy should focus on another *C. albicans* extracellular matrix components besides EPS-soluble and-insoluble, such as eDNA and proteins.

Therefore, we conclude that *C. albican*s biofilms biomasses (dry-weight) were significantly reduced by the treatment with red and blue light. Moreover, the EPS-soluble content was mainly reduced by twice-daily exposure to blue light while EPS-insoluble exhibited major reduction with twice-daily treatment with red light for 1 min. This leads us to believe that light is a promising therapeutic approach for biofilm-related *C. albicans* diseases, such as oral candidiasis, indicating that twice-daily treatment of *Candida* biofilms with either blue light and red light can function as an adjuvant to topic antifungal application.

Aknowledgment

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Conflict of Interests

None.

Ethical Considerations

This article does not contain any studies with human or animal subjects performed by any of the authors.

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3.2 Capítulo 2:

Antimicrobial effect of Low Temperature Plasma on oral biofilm formed *in situ*: molecular partial identification of microbial population

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Abstract

Objectives To evaluate the antimicrobial effect of tissue tolerable plasma on oral biofilms formed in situ through the molecular characterization of the microbial population.
Material and methods For this *in situ* experiment, a single-blind split mouth design was used in two phases of 7 days each, in which 8 volunteers wore palatal devices containing six bovine enamel slabs, positioned in pair of three. At the end of the clinical phase, the device was randomly split and each half was allocated to one of the following treatments: Plasma (PLA); Argon (ARG); Chlorhexidine 0.12% (CHX) and Salina solution 0.89% (NaCl). In this way, at the end of the two clinical phases, biofilms form all volunteers were submitted to the four different treatments.
Results A total of eight samples from each group were submitted to the RT-q PCR for the bacteria detection and quantification. Data of the gene expression of each bacterium were obtained. Statistical analysis were performed using the Sigma Plot program, using Anova test, followed by the complementary tests of Student t-test and Newman Keuls, with $p < 0.05$. In all the analyzed groups the presence of specific bacteria for each primer was observed, however some groups treated with plasma the bacterial expression of the bacteria was lower. Plasma treatment on biofilm sample presented significantly lowers concentrations of *Streptococcus mutans*, *Lactobacillus acidophilus*, *Streptococcus mitis* group, *Bifidobacterium* group and the *Actinimyces naeslundii* ($p < 0.05$) compared to other treatment groups. Concentrations of *Lactobacillus casei* groups and the *Streptococcus gordonii* were not significantly different before and after plasma treatment.
Clinical relevance Considering that the information about the oral microbiota related to biofilm *in situ* status is relevant, this study provides insights to better understand the differences in the microbiotas between different treatments.
Keywords Bacteria- biofilm- *in situ*- Quantitative polymerase chain reaction

Introduction

The impact of environmental factors on chronic diseases has been the focus of many studies, especially in the last couple of decades, along with an effort to understand the heterogeneity of the immunological parameters among individuals [1]. In between different environmental factors, one that stands out is the microbiota that may be defined as the sum of microbes residing in a habitat. The oral cavity is the habitat of several kinds of microorganisms, which form a complex community that can adhere to the teeth surface or to epithelial mucosa forming biofilms [2]. The microbiome is defined as the totality of genes of microbiota, in this case, of that oral microbiota [3]. In oral health, the oral cavity microbiome comprises billions of microbes.

Oral microbial biofilms are three-dimensional structured bacterial communities [4] attached to a solid surface like the enamel of the teeth, the surface of the root or dental implants [5] and are embedded in an exo-polysaccharide matrix [6]. Oral biofilms are exemplary and served as a model system for bacterial adhesion [7,8] and antibiotic resistance [9]. The bacterial diversity in the oral cavity is estimated to be more than 700 different species and phylotypes, being that of these, 50% are cultivable and the rest indentifiable only by molecular biology [10]. Among these microorganisms, *Streptococci* species comprise the majority of dental plaque population [11]. The original mechanistic analyses revealed that the demineralization of the enamel was induced by the increasing acidity of the microenvironment surrounding the tooth. This acidity was mainly

generated by a selective group of bacteria [12]. The advent of molecular research that characterizes the oral microbiota in health and disease revealed the diversity of oral biofilms, introducing new candidates for disease-associated bacterial species [13]. Molecular approaches have revealed a greater variability of the oral microbiota associated to dental caries, including *Streptococcus spp* and bacteria of the genera *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Veillonella*, *Selenomonas*, and *Atopobium* [14].

It is well know that the accumulation of bacterial biofilms on tooth surfaces results in some of the most prevalent bacterial-induced human diseases, caries and inflammatory periodontal diseases. Current treatment of subjects with plaque-related diseases involves mechanical removal of the biofilm and the use of antiseptics and antibiotics. Thus, the increased microbial resistance against commercially available antimicrobial drugs and substances have cooperated with the search for alternative treatments for the control of pathogenic biofilms involved with diseases that affect the body, including biofilm-dependent oral diseases [15, 16]. In this context, plasma may constitute a suitable process to combat both biofilm-related resistance and antimicrobial resistance.

Plasma is a partially ionized gas generated by an electrical discharge, which creates a highly reactive environment with ions, electrons, excited atoms and molecules, vacuum ultraviolet and ultraviolet (UV) irradiation, free radicals, and chemically reactive particles [17]. It is also specific, targeting only the infected area. In addition, plasma is usually produced by low-toxicity gases and elaborates its activity by producing a mixture of products that decay within a few seconds after the treatment process [18] and the ability to achieve gas phase, without the need to reach high temperatures, allows its use in thermosensitive materials including cells and tissues [19]. The effectiveness of removing biofilms and inactivation of microorganisms with tissue tolerable plasma have been demonstrated [20].

In general, biofilms models may help us to accurately predict, in a controlled and simplified way, a clinical outcome which can lead us to preventive actions for diseases [21]. In this way, we decided to evaluate the effect of plasma under conditions more similar to those found in the mouth, using an *in situ* multispecies biofilm model. Method involves the use of devices that create conditions reproducing the process of biofilm formation in the oral cavity, serving as a link between the uncontrolled clinical situation and the highly controlled laboratory experiments.

The primary purpose in this study is to evaluate the antimicrobial effect of tissue tolerable plasma on oral biofilms formed *in situ* through the molecular identification of the part microbial population.

Methods

Ethics statement

This study protocol was approved by the Research and Ethics Committee of the Federal University of Ceara, Brazil Medical School (Sisnep Caae - 40975514.0.0000.5054). All volunteers gave informed consent according to Resolution n^0 196 of the National Health Council, Health Ministry, Brasilia, DF, from 10/03/1996.

Experimental design

For this *in situ* experiment, a single-blind split mouth design was used in two phases of 7 days each, in which 8 volunteers wore palatal devices containing six bovine enamel slabs, positioned in pair of three. At the end of the clinical phase, the device was randomly split and each half was allocated to one of the following treatments: Plasma (PLA); Argon (ARG); Chlorhexidine 0.12% (CHX) and Salina solution 0.89% (SAL) as described in Fig 1. In this way, at the end of the two clinical phases, all volunteers were submitted to the four different treatments.

Figure 1. Description of the treatments in which the enamel slabs were treatds

Groups	***Code***	***Treatment***
Plasma	*PLA*	Plasma plume during 5 min
Argon	*ARG*	Argon gas flow during 5 min
Chlorhexidine 0.12%	*CHX*	*50* μL on each slab during 5 min
Salina solution 0.89%	*SAL*	*50* μL on each slab during 5 min

Tissue tolerable plasma

The Atmospheric-Pressure Plasma (plasma jet kINPen med®) that was utilized in this study was developed by the Leibniz Institute for Plasma Science and Technology (Neoplas Tools – Kinpen, Greifswald, Germany) and consists of a hand-held unit (length = 170 mm, diameter = 20 mm, weight = 170g) connected to a high-frequency power supply (frequency 1.82 MHz, 2–6 kV peak-to-peak, 8 W system power) for the generation of a plasma jet at atmospheric pressure. The handheld unit has a pin-type electrode (1 mm diameter) surrounded by a 1.6 mm quartz capillary. An operating gas consisting of argon at a flow rate of 5 slm (standard liters per minute) was used. The plasma plume emerging at the exit nozzle is about 1.5 mm in diameter and extends into the surrounding air for a distance of up to 10mm. The gas flow plume was targeted to all surface of

enamel slabs, and the tip-to sample distance (10 mm) was kept constant during application using a fixed holder.

Specimen preparation

Bovine teeth were used to perform this *in situ* study. The teeth were stored in 0.01% (v/v) thymol solution at 4°C for 30 days until used [21,22,23]. Enamel slabs with 4 x 4 x 2 mm were obtained using a water-cooled diamond saw and a cutting machine (IsoMet Low Speed Saw; Buehler, Lake Bluff, IL, USA). The adjustment of the enamel and dentin to obtain flat plates was done with the aid of a low-speed polishing machine and 320 grit paper (Carbimet Paper Discs), under water-cooling. Afterwards, the specimens were polished using three different silicon carbide waterproof papers (320, 600, and 1,200-grit) as well as polishing cloths with 1 μm diamond paste (Buehler).

In situ palatal devices

The slabs were autoclaved (121 ºC, 15 min) [24] and stored in 100% humidity until being inserted into the palatal appliances. For each subject, two acrylic palatal devices were fabricated, in which two cavities (18 x 6 x 3 mm) were prepared on the left and right sides; three slabs were attached with wax in each cavity. In order to allow biofilm accumulation, and to protect it from mechanical disturbance, a plastic mesh was positioned on the acrylic resin, leaving a 1 mm space from the slab surface [25,26].

In situ study Population

Eight healthy volunteers (5 women and 3 men), aged 19–34 years, able to comply with the experimental protocol, were selected to participate in this study. All participants received oral and written instructions about the experimental design. The inclusion criteria were normal salivary flow rate, normal buffering capacity of saliva and *S. mutans* colony-forming units (CFU mg^{-1}) in biofilms of at least 10^5 after 36 h of oral hygiene suspension. Exclusion criteria included active caries lesions, use of antibiotics within the past 3 months prior to the study, use of fixed or removable orthodontic devices. The use of dentifrice containing any antibiotics was suspended during the experimental period.

In situ biofilm formation

During the lead-in period (7 d) and throughout the clinical phases (7 days each), the volunteers brushed their teeth with a fluoridated dentifrice [Sorriso Super Refrescante – a calcium

carbonate based dentifrice, 1,450 µg fluoride (F) g^{-1}, as monofluorophosphate (MFP); Colgate-Palmolive, São Paulo, SP, Brazil]. Also, the volunteers received oral and written instructions to wear the appliances at all times, including at night. They were allowed to remove the appliances only during meals, when consuming acid drinks, and when performing oral hygiene. When removed, the devices were kept moist in plastic boxes to keep the bacterial biofilm viable [23]. The cariogenic challenge was provided by the volunteers who dripped a 10% sucrose solution onto all the enamel slabs, 10 times a day, according to a predetermined schedule (at 08:00, 09:30, 11:00, 12:30, 14:00, 15:30, 17:00, 18:30, 20:00, and 21: 30 h) [29]. Before replacing the palatal appliance in the mouth, a 5-min waiting time was standardized to allow diffusion of the sucrose into the dental biofilm. Brushing with the dentifrice was performed three times a day, after mealtimes when the volunteers habitually carried out their oral hygiene procedures. The appliances were brushed extra-orally, except for the slab area, and volunteers were asked to brush carefully over the covering meshes, to avoid disturbing the biofilm. All volunteers consumed fluoridated water (0.70 mg F 1^{-1}), and no restriction was made with regard to the volunteers' dietary habits.

Plasma treatment of in situ biofilms

The distribution of treatments on the palatal device in each intra-oral phase was determined randomly by raffle. All volunteers came in fasting, removed the device from mouth and one drop of 10% sucrose was added to each slabs. Third minutes later the plastic meshes of the devices were removed with a scalpel blade (#15C), the biofilm formed *in situ* were exposed, and the treatments with PLA, ARG, CHX or SAL were performed. Biofilms were then scraped carefully, were and weighed and were suspended in RNAlater solution adding 5–10 volume. Samples were stored at room temperature overnigh and stored at -80 ^{o}C after that.

Extraction of RNA

The biofilm samples (8 samples / group) were initially thawed. The RNAlater solution was removed by washing with PBS and then transferred to threaded cryotubes (Axygen, Union City, CA, USA), which previously received 0.16 g of 0.1 mm diameter zirconia beads [30]. The tubes were shaken in a Beadbeater apparatus (Biospec Products Inc., Bartlesville, OK, USA) for two 30-second periods, with a 60-second immersion of the tube on ice for each shaking period. This procedure aimed at breaking the bacterial cell wall and consequently releasing the nucleic acid molecules.

RNeasy Minikit

After stirring at Beadbeater, 850 µl of a mixture of RLT (*RNeasy* Minikit ®) and 1% β-mercaptoethanol was added to the biofilm sample, vortexed for 30 seconds and centrifuged for 2 minutes (11000g / 4 ° C). Thereafter, 350 µl of the supernatant was removed for the continuation of the extraction procedure, while the other part was stored if further extraction was required. To the 350 µl initially removed, 250 µl of pure ethanol was added and vortexed. Then, the contents were transferred to a RNeasy MiniKit column (Qiagen, Valencia, CA, USA) and centrifuged. After centrifugation, the contents that had passed through the column were discarded. 700 µl of RW (*RNeasy* Minikit ®) were added to the columm and again centrifugation (11000g / 20 ° C) for 30 seconds was performed. The content that had passed through the column was discarded again. Thereafter, 500 µl of RPE (*RNeasy* Minikit ®) was added to the column and centrifuged again (11000g / 20 ° C) for 30 seconds. This step was performed twice. A centrifugation without addition of reagents (11000g / 20oC) for 2 minutes to remove all alcohol present on the column was performed. Subsequently, the column was placed in a capped eppendorf and 40 µl of RNAse free water was added and centrifuged again (11000g / 20 ° C) for 1 minute to elute. The eluate was pipetted and stored in a new labeled eppendorf.

Treatment with DNase

For each RNA sample obtained, 5 µl of the buffer and 5 µl of the Turbo DNase enzyme (Applied Biosystems, Ambiom, Austin, TX, USA) were added. After vortexing, the sample was allowed to stand for 15 minutes at 37 ° C, sufficient time for DNA degradation by DNAse.

Purification of samples

To purify the samples, 300 µl of RW buffer (*RNeasy* Minikit ®) and 250 µl of pure ethanol were added to the column, vortexing and rapid spin were performed. The contents were transferred to a new RNeasy MiniKit column, centrifuged (11000g / 20oC) for 30 seconds, and what went through the column was discarded. Then the column 700 µl of RW was added, centrifuged again (11000g / 20 ° C) for 30 seconds and the contents passed through the column were discarded. 500 µl of the RPE buffer was added to the sample, and the sample was again centrifuged (11000g / 20 ° C) for 30 seconds and the contents that passed through the column were discarded. This step was repeated twice. A further centrifugation was performed for 2 minutes so that excess ethanol was removed. After removal of excess ethanol, the column containing 30 ul of ultrapure water was transferred to a capped eppendorf tube and elution of the RNA by another centrifugation (11000g / 20 ° C) for 1 minute was performed. Total extracted and purified RNA from the sample was stored on ice for immediate processing.

Dosage and integrity of total RNA

The amount of total RNA extracted (ng/µl) was measured in Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) (A260 / A280 ratio). Then, the integrity of this RNA was verified through an electrophoresis gel run.

Reverse transcriptase reaction and cDNA uptake

The cDNA was produced using the iScript ™ cDNA Synthesis kit (BioRad, Hercules, CA, USA). Reverse transcriptase reactions were prepared from a mixture containing 6 µl of the iScript 5x reaction mix, 1 µl iScript reverse transcriptase, 1 µg of total RNA extracted from the biofilm sample and sufficient RNAse free water to complete a volume of 30 µl. Each prepared solution was vortexed for 5 seconds, incubated at 25° C for 5 minutes, heated at 42 ° C for 2 hours, and again heated at 85 ° C for 5 minutes in a Veriti Thermal Thermal Cycler (Applied Biosystems, Foster City, CA, USA). After all reactions, the cDNA concentration of all samples were adjusted to 10 ng / µl (Bezerra et al., 2016).

Real-time polymerase chain reaction

The quantitative real-time PCR technique (qRT-PCR) was performed using StepOneTM Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed using 48-well plates coated with adhesive film (Applied Biosystems, Foster City, CA, USA). Each well was filled with a solution prepared with 5 µl of master mix SYBR Green (iQTM SYBR Green Supermix, Applied Biosystem, Foster City, USA), 2.4 µl of nuclease-free water, 0.3 µl of each primer F / R (table 1) 10 µM and 2 µl cDNA (20 ng) of each sample. The assays were performed in duplicate, and the final analyzes were based on the mean of the two reactions. Standard curves were performed for each primer (Table 1). The presence of genomic DNA contamination was determined by the accomplishment of control reactions without the addition of reverse transcriptase.

Table 1. *Bacterial identification primers used in Qrt-PCR*

SPECIES	TARGET	SEQUENCE (5' 3')	ANNEALING TEMPERATURE (°C)	AMPLICON LENGTH (BP)
Bacteria 16S rDNA	BT	F: TCCTACGGGAGGCAGCAGT R:GGACTACCAGGGTATCTAATCCTGT	57	466
23S rRNA[1]	ST	F: AGCTTAGAAGCAGCTATTCATTC R: GGATACACCTTTCGGTCTCTC	60	318
Actinimyces naeslundii	AN	F:CTGCTGCTGACATCGCCGCTCGTA R:TCCGCTCGCGCCACCTCTCGTTA	62	144
Bifidobacterium spp.[1]	BB	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	58	243
Lactobacillus acidophilus	LA	F: GATCGCATGATCAGCTTATA R: AGTCTCTCAACTCGGCTATG	60	124
L. casei group[2]	LC	F: GCGGACGGGTGAGTAACACG R: GCTTACGCCATCTTTCAGCCAA	60	121
Mitis group[3]	MG	F:TAGAACGCTGAAGGAAGGAGC R: GCAACATCTACTGTTATGCGG	60	133
Streptococcus gordonii	SG	F: CAGGAAGGGATGTTGGTGTT R: GACTCTCTTGGCGACGAATC	60	136
Streptococcus mutans	SM	F: AGCCATGCGCAATCAACAGGTT R: CGCAACGCGAACATCTTGATCAG	64	415

1. Bifidobacterium longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminantium, B. thermophilum, B. subtile, B. bifidum, B. boum, B. lactis, B. animalis, B. choerinum, B. gallicum, B. pseudolongum subsp. globosum, B. pseudolongum subsp. pseudolongum, B. magnum, B. infantis, B. indicum, B. gallinarum, B. pullorum, B. saeculare, B. suis
2. L . casei group: L. casei, L. paracasei, L. rhamnosus and L. zeae.
3. Mitis group : Streptococcus mitis, S. oralis, S. pneumoniae, S. parasanguinis, S. australis

Statistical analysis

Based on the study by Arthur et al. (31,32), a sample of 8 units per study group was estimated, with a power of 90% and confidence of 95% (test t, www.Openepi .com/samplesize/ssmean.htm). Eight samples were used for each group and for each gene analyzed in this study. Data were tabuleted in Microsoft Excel and exported to a statistical software Sigma Plot on the results were analyzed using the Kolmogorov-Smirnov test. Statistical was performed using the sigma plot program, using anova, followed by the complementary tests os student and Newman Kells, with $p< 0,05$. Data were expressed as the mean and standard deviation and compared between groups. For bacterial prevalence data, the values of the concentration of the bacterial species werw calculated as a percent of the total load.

Results

A total of eight biofilm samples were collected for each treatments. Biofilm samples were divided into four groups: PLA (n = 8), SAL (n = 8), ARG (n=8) and CHX (n=8). The concentrations of each strain were obtained by normalization to total bacteria present in the same biofilm sample as determined using specific primers. Table 2 details the mean and median values of the prevalence of oral bacteria in each treatment.

Plasma treatment on biofilm sample presented significantly minors concentrations of *S. mutans*, *L. acidophillus*, *S. mitis* group, *A. naeslundis* and the *Bifidobacterium* group ($p < 0.05$) compared to other treatment groups. Concentrations of *L. casei* groups and the *S. gordonii* were not affected by different treatments. In all the analyzed groups the presence of specific bacteria for each primer was observed, however some groups treated with plasma the bacterial expression of the bacteria was lower.

Table 2: Relative concentrations of oral bacteria in groups samples as determined by qPCR and normalized by total bacteria

Oral bacteria	Groups	Mean	±SD	Median	p Value
S. mutans	PLA	0.000000127	7.51E-08	-	0.024*
	SAL	0.000000517	0.000000153	-	
	ARG	0.000000223	7.26E-08	-	
	CHX	0.000000181	0.00000027	-	
L. acidophillus	PLA	-	0.00000277	0.000000792	0.021*
	SAL	-	4.1	0.0000179	
	ARG	-	0.00000262	0.0000414	
	CHX	-	0.000028	0.00000507	
S. mitis group	PLA	0.24	0.206	-	0.009*
	SAL	4.507	1.483	-	
	ARG	1.684	0.372	-	
	CHX	1.12	1.646	-	
L. casei group	PLA	0.0288	0.0297	-	0.511
	SAL	0.288	0.375	-	
	ARG	0.312	0.279	-	
	CHX	0.349	0.0306	-	
Bifidobacterium spp	PLA	-	0.0525	0.00332	0.042*
	SAL	-	0.357	0.305	
	ARG	-	0.0614	0.0419	
	CHX	-	0.0187	0.028	
A. naeslundii	PLA	-	0.0000308	0.00000393	0.029*
	SAL	-	0.0000546	0.0000364	
	ARG	-	0.0000265	0.00000852	
	CHX	-	0.0000222	0.0000237	
S. gordonii	PLA	0.00000754	0.0000128	-	0.606
	SAL	0.0000221	0.0000326	-	
	ARG	0.00000622	0.00000682	-	
	CHX	0.00000298	0.00000267	-	

Additionally, the presence and absence of the bacteria of some groups are summarized in the figures 1, 2, 3, 4, 5, 6 and 7.

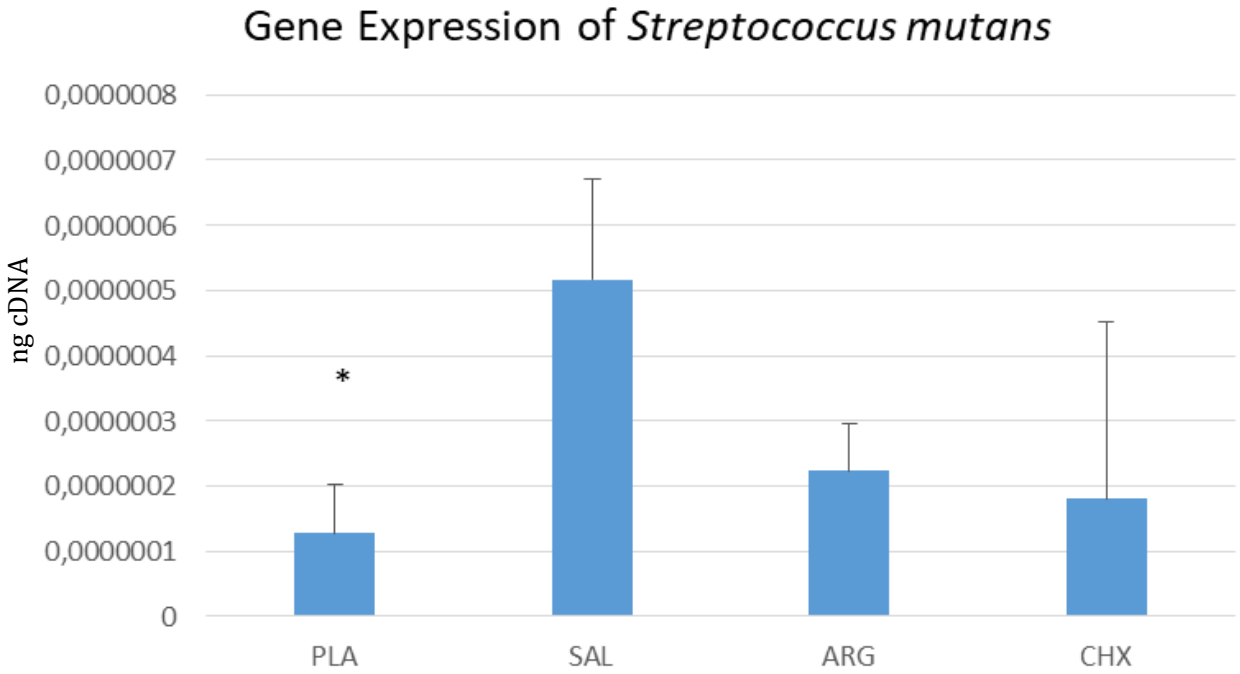

Figure 1: Relative concentrations of oral bacteria in groups to *S. mutans* as determined by qPCR and normalized by total bacteria. The * points to significant differences ($p<0.05$) in comparison to the other groups.

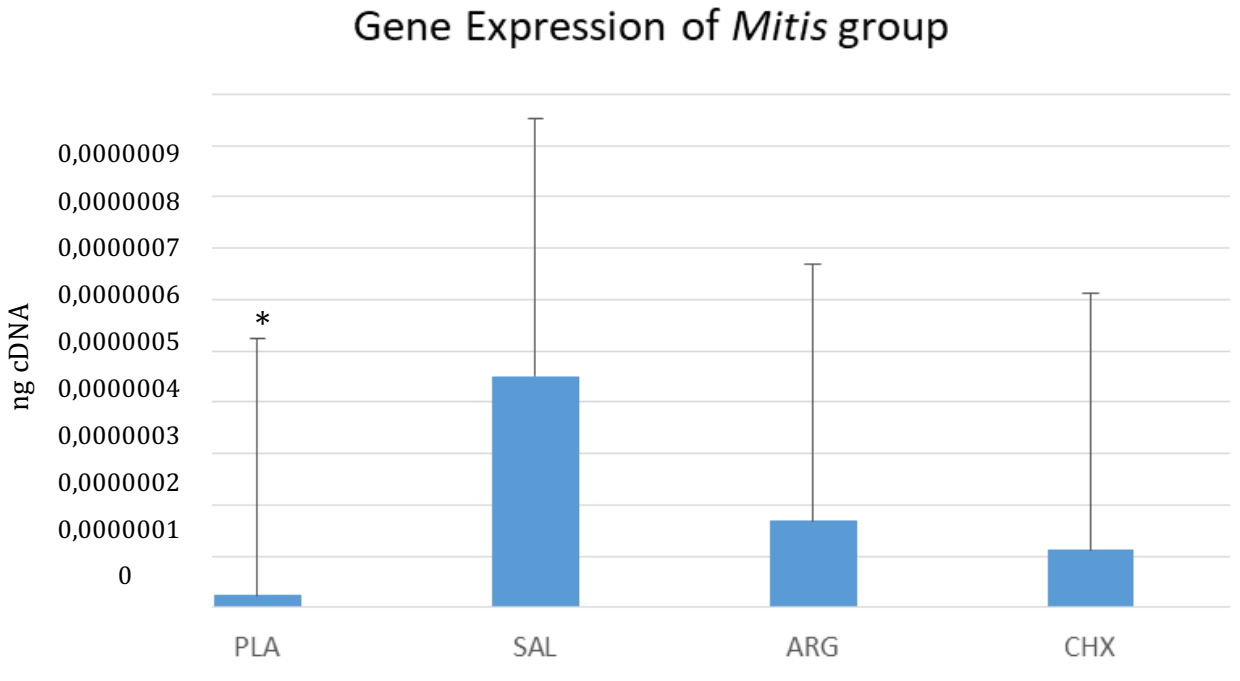

Figure 2: Relative concentrations of oral bacteria in *Mitis groups* as determined by qPCR and normalized by total bacteria. The * points to significant differences ($p<0.05$) in comparison to the other groups.

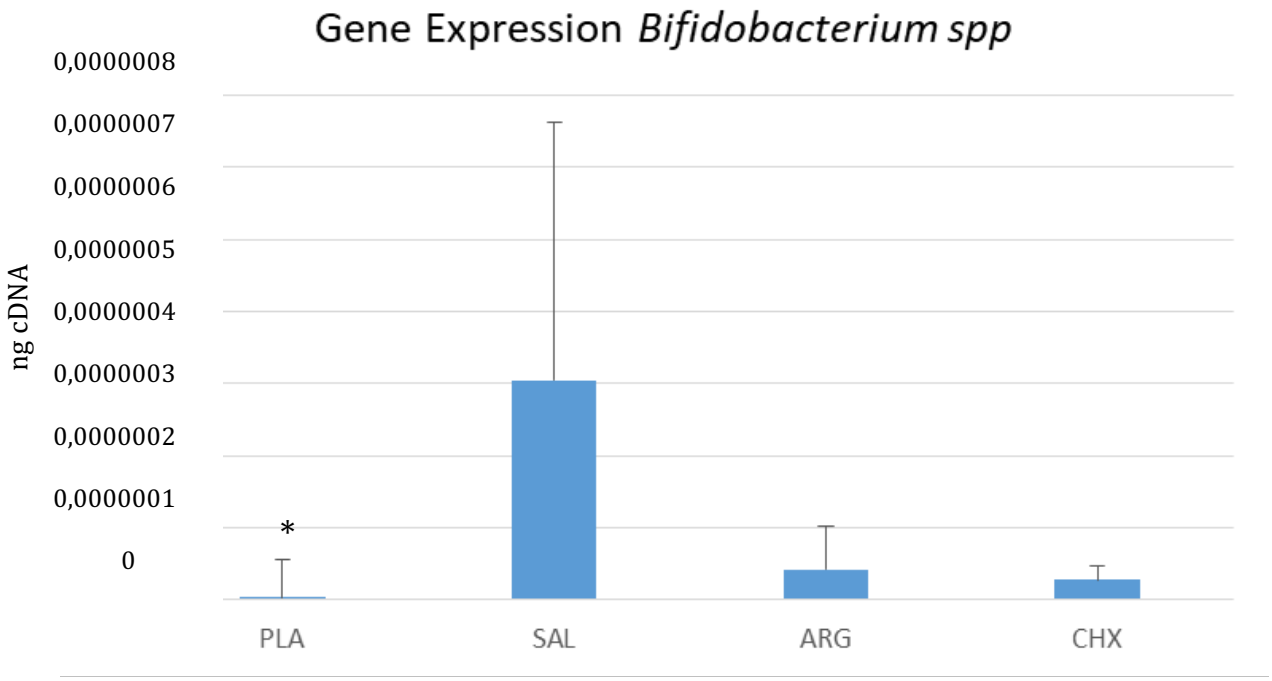

Figure 3: Relative concentrations of oral bacteria in groups to *Bifidobacterium* as determined by qPCR and normalized by total bacteria. The * points to significant differences ($p<0.05$) in comparison to the other groups.

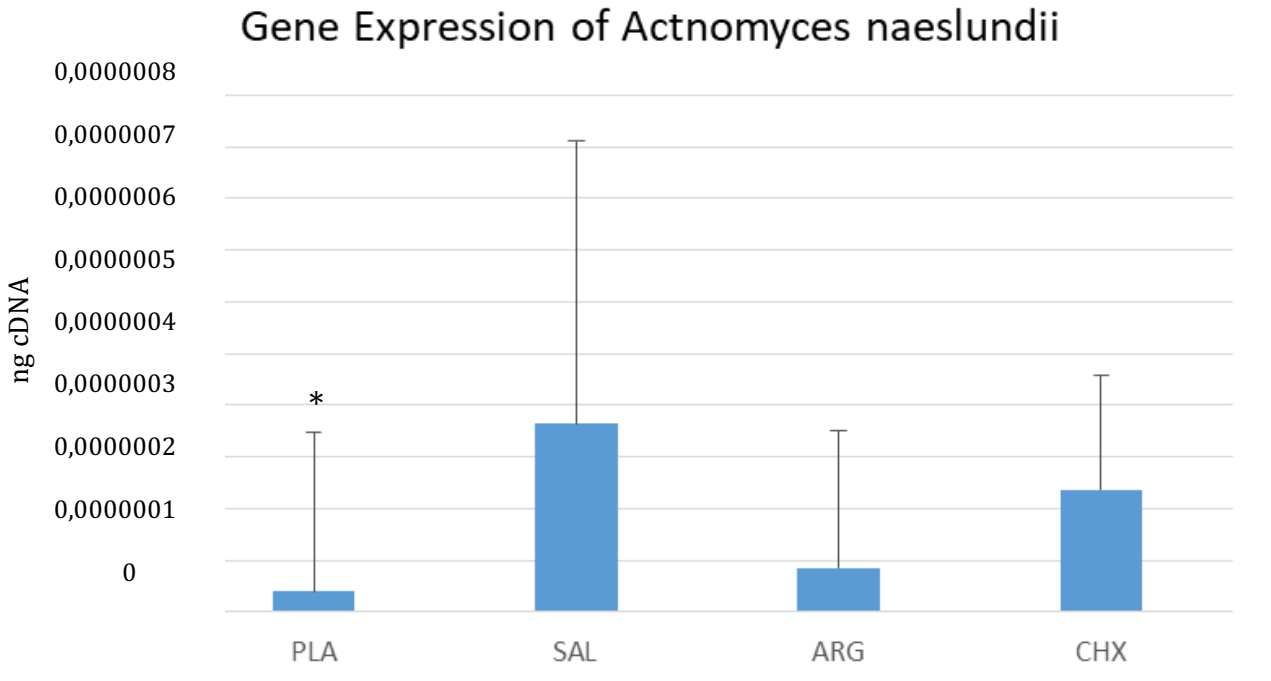

Figure 4: Relative concentrations of oral bacteria in groups to *A. naeslundii* as determined by qPCR and normalized by total bacteria. The * points to significant differences ($p<0.05$) in comparison to the other groups.

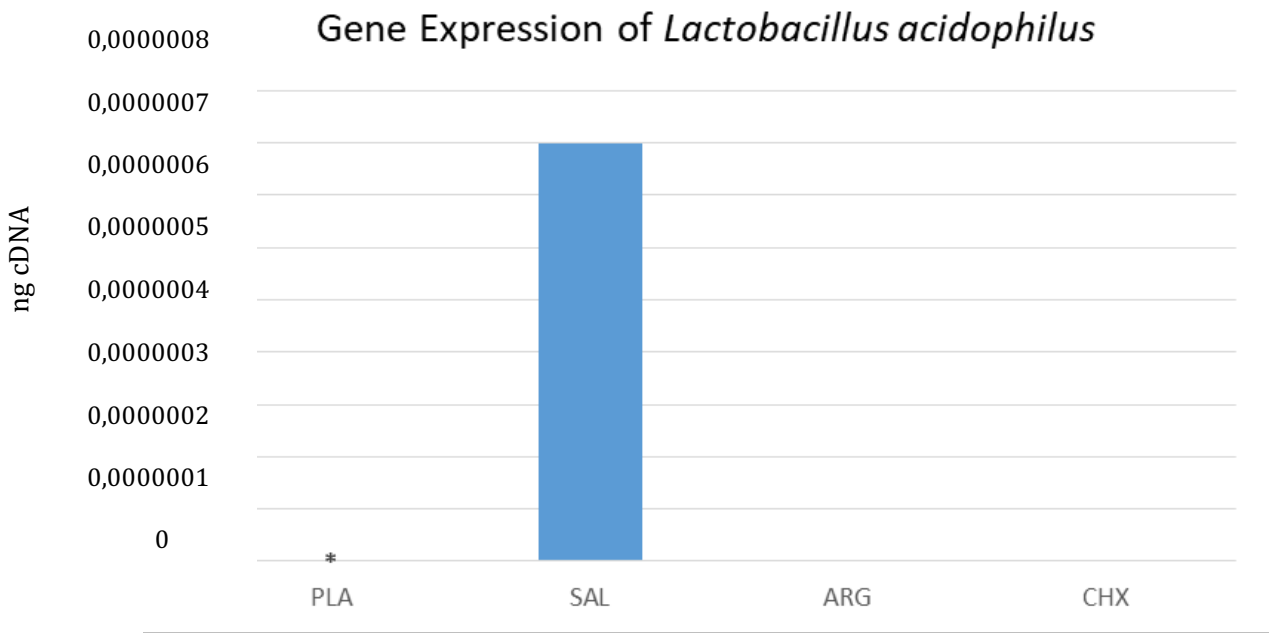

Figure 5: Relative concentrations of oral bacteria in groups to *L. acidophillus* as determined by qPCR and normalized by total bacteria. The * points to significant differences ($p<0.05$) in comparison to the other groups.

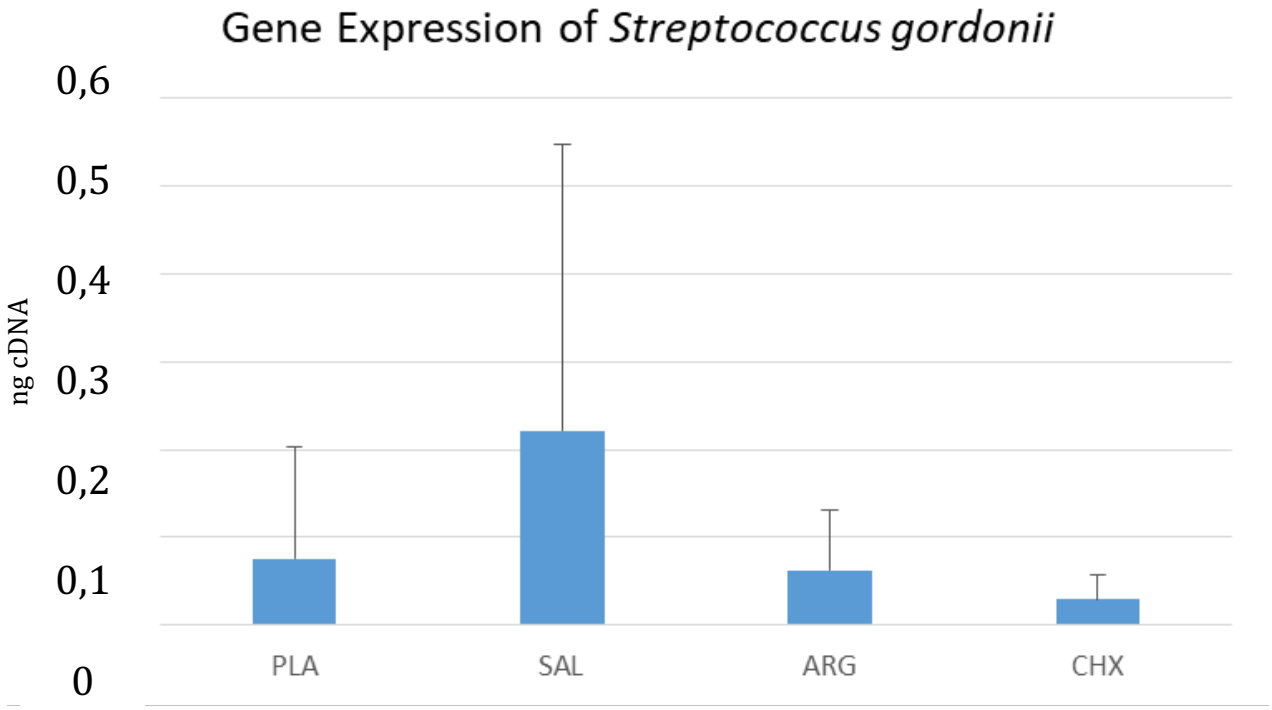

Figure 6: Relative concentrations of oral bacteria in groups to *S. gordoni as* determined by qPCR and normalized by total bacteria.

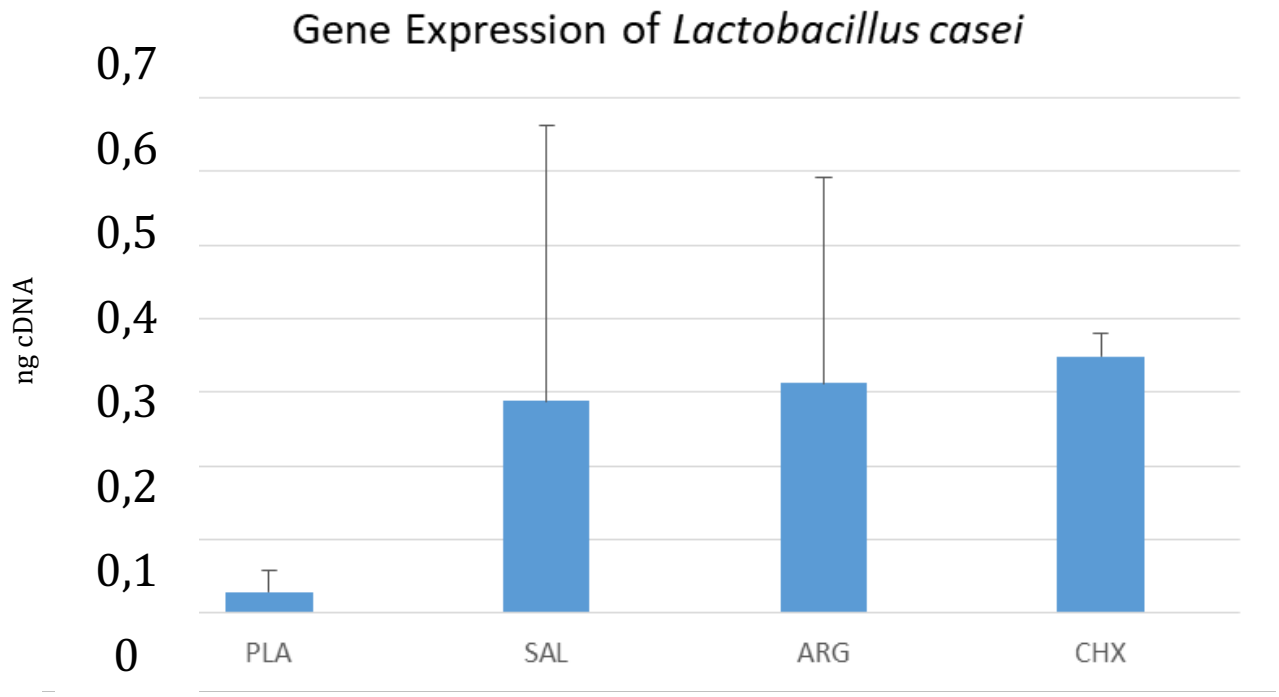

Figure 7: Relative concentrations of oral bacteria in groups to *L. casei group* as determined by qPCR and normalized by total bacteria.

Discussion

The main finding of the present study is that plasma was effective against mature oral biofilm formed *in situ*, based of the gene expression being, in some cases, more effective in reducing bacterial viability than chlorhexidine, a well-known antimicrobial substance. The oral biofilm containing pathogenic bacteria communities is one of the major factor associated with oral disease [33]. Consequently, the interest in new strategies to effectively inactivate pathogenic bacteria in oral biofilms has emerged in the scientific community and the use of plasmas is one of these new therapies due to its effectiveness against oral microorganisms [34, 35, 36, 37, 38, 39, 40].

This study quantified the gene expression in biofilm of the antimicrobial effect of several treatments on oral biofilms. CHX solution is the gold standard treatment to inactivate or prevent dental plaque formation [41] when compared to other chemical agents used in dentistry [42]. The main advantage of using CHX is its wide antimicrobial spectrum, acting on both Gram-positive and Gram-negative microorganisms and its prolonged and continuous effect even in the presence of blood and other body fluids [43]. However, the prolonged use of CHX can cause mucous peeling, stains on the teeth, alterations in the sense of taste, compromising of the healing process and reduction of fibroblast adhesion to radicular surfaces [44].

In our study, plasma treatment showed better results than CHX in gene expression of many bacterias, similarly found by the Koban et al. [45] *in vitro* using plasma treatment against oral

biofilms formed on titanium discs. Also, these results are in accordance with the literature in that a 0.1% CHX solution is inefficient against mature oral biofilms [46].

Our findings indicate that *S. mutans*, *L. acidophillus*, *S. mitis* group, *L. casei* group, *Bifidobacterium spp*, *A. naeslundii* and *S. gordoni* are part of the viable microbiota. Gene expression analysis suggests that the quantification of this microoganisms may differ according to biofilm treatment. Despite the evidence of the presence of bacteria, its virulence may be affected by environmental changes and the diferent treatements [47,48].

Real-time PCR was the chosen method for this study, since the use of qPCR is an accepted technology for the quantitative analysis of bacteria from mixed samples. Furthermore, this methodology allows the microorganisms to be assessed more accurately than they can be by cultural analysis. Quantitative PCR has the potential to account for the uncultivable portion of the oral microbial community, as well as, species which are more difficult to culture [49]. However, it seems that this study presents important findings, since differences in the microbiotas of each tretament have not yet been elucidated, according to Takahashi & Nyvad [51].

Initial plaque formation starts with the deposition of a salivary pellicle on the tooth surface. Planktonic cells or aggregates of cells adhere to this pellicle via specialized adhesins on the bacterial cell surface that recognize pellicle proteins [52]. These phenomena may result in a scattered pattern of bacterial deposits [53,54] composed of initial colonizers like *Actinomyces sp*, *Streptococcus sp*, and *Lactobacillus sp* and it is reflected in the different biofilm types.

Our findings indicate that presence of this microorganisms, however, the plasma treated groups identified a statistical difference for the *Actinomyces naeslunddi*, *Streptococus mutans* and *Mitis* and for the *Acidophillus lactobacillus* in comparison to the other treatments (Table 2), indicating important plasma performance in the basins that act of biofilm formation.

Streptococcus mutans has been strongly implicated as the main etiological agent in human dental caries [55]. One of the important virulence properties of these organisms is their ability to form biofilms known as dental plaque on tooth surfaces [56]. Dental plaque is one of the best-studied biofilms [55]. Dental plaque formation on tooth surfaces involves three distinct steps: (i) formation of the conditioning film or acquired pellicle on the tooth enamel, (ii) subsequent cell-to-surface attachment of the primary colonizers, and (iii) cell-to-cell interactions of late colonizers with one another as well as with the primary colonizers [55].

The colonization of tooth surfaces by *S. mutans* appears to result from two distinct processes: initial sucrose-independent attachment and enhancement of attachment by sucrose-dependent mechanisms involving [57]. The role of sucrose and Gtfs in *S. mutans* biofilm formation has been well documented [58,47]. In addition, several other genes associated with biofilm formation have been reported in recent investigations [59].

In our study, a significant difference was identified in the presence of *S. mutans* in the plasma treated group in relation to the other groups. According to what has been reported in relation to this bacteria, this is an important factor for the control of dental biofilm.

The recent field of plasma medicine is a rapidly growing and innovative interdisciplinary endeavor encompassing plasma physics, life sciences, biochemistry, engineering and clinical medicine [60]. An important feature of non-equilibrium (cold) APP is its ability to produce a mixture of biologically active agents, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), while remaining close to ambient temperature, which enables its safe application to living cells and tissues.

Like the *S. mutans*, it is known that the presence of *Lactobacilli* occurs in high numbers in superficial and deep caries [65]. *Lactobacillus acidophilus* are usually numerically dominant, although *Lactobacillus paracasei*, *Lactobacillus Rhamnosus* and *Lactobacillus fermentum* are also present. The findings in our study also identified a significant difference between the biofilm treated with plasma and the other treatments in the quantification of *L. acidophilus*. *Lactobacillus acidophilus* and *Streptococcus mutans* are common Gram-positive oral bacteria responsible for causing oral caries [66,67]. In a previous study, atmospheric plasma was found to be effective in deactivating bacterium when seeded on glass, filter paper, and PTFE supporting media [37].

Sladek et al. [40] reported on the feasability of the application of plasma to treat dental caries. They evaluated the temperature increase in the pulp of a tooth during plasma treatment, and the possibility of plasma as a substitute for rotary instruments. The temperature increase in the pulpal chamber was ~2.3 C during the plasma treatment of the enamel surface. The same study group also confirmed the capability of the plasma device for killing bacteria [35]. Yang et al. [37] reported on the bactericidal effect of a non-thermal atmospheric pressure plasma brush on *S. mutans* and *L. acidophilus*, which are major pathogens in dental caries. Although plasma could not replace rotary instruments, it is expected that plasma will play important roles in the prevention and treatment of dental caries.

The presence of *Actinomyces naeslundii* was identified in the biofilm samples that were studied. The presence of *A. naeslundii* is associated with biofilm formation. Several Actinomyces species belong to the resident oral microbiota of supra-gingival plaque, although studies based on culture, checkerboard hybridization, 16S rRNA gene libraries and FISH show significant differences in their proportions depending on the age of the biofilm [68, 54, 69, 70]. Dige et al [71] using a species-specific oligonucleotide probe confirms the checkerboard hybridization-based demonstration of *A. naeslundii* as a significant member of the initial colonizers of tooth surfaces and demonstrates that *A. naeslundii* preferentially occupies the inner part of early multilayered biofilms. In this Dige et al study (2009), *A. naeslundii* was frequently observed in mixed groups

with streptococci and other bacteria. This observation supports the view that co-adhesion, in particular the co-adhesion processes involving *A. naeslundii*, *streptococci* and other bacteria, plays an important role in the early stages of colonization of dental surfaces [7,72, 2, 53,73]. This observation is further supported by the discovery of genotypically different bacteria located on the outer surface of the biofilm, indicating that the co-adhesion of saliva bacteria is a continuous process that increases the biomass of the developing biofilm.

Actinomyces species can use lactate as a carbon source for growth [74, 75], whereby lactic acid is converted into weaker acids [76]. A pH-modulating activity of these species may, theoretically, occur also via degradation of urea [77]. Moreover, through its metabolism, Actinomyces species can remove oxygen from the environment and create an anaerobic milieu [51], suitable for the outgrowth of some other bacteria. Finally, recent observations demonstrate that co-aggregation with *A. naeslundii* stabilizes arginine metabolism in *S. gordonii* and reduces its dependence on extracellular arginine, which is a limiting factor in the environment of the early colonizers [78,79]. Collectively, these properties make *A. naeslundii* an essential initial colonizer of tooth surfaces and particularly well adapted to live and survive in substrate-limited environments deep in the biofilm. The fixed metabolic activities of these bacteria may have a controlling effect on dental caries processes by reducing the acidogenic potential of the biofilm [51] Therefore, this finding in our study is very important because in our study the treatment of plasma was effective in relation to the other treatments regarding the identification of *A. naeslundii* in the samples.

In addition, although members of the *S. mitis* group were previously detected in carious lesions [61,62], and in this study we found a diference between the groups, the *S. mitis* group have been frequently associated with health [63,64], making the contribution of these bacteria to biofilm inactivation.

Cold plasma has emerged as a physical treatment with microbicidal effectiveness on bacteria, parasites, fungi, spores, and viruses [80]. In the present study, the antimicrobial effect of plasma was confirmed, since all plasma-treated samples exhibited significant lower viability than positive and negative controls. Delben [81] suggested that the reactive oxygen species (i.e. ozone, atomic oxygen, superoxide, peroxide, hydroxyl radical, and nitric oxide) produced by plasma generated oxidative effects in cellular biomacromolecules including DNA, lipids and proteins [18]. As a consequence, oxidative stress causes lipid peroxidation and oxidation of several amino acids of proteins, which compromises the function and integrity of membrane and cell wall [82,83]. In addition, membrane desestabilization affects the ability to maintain proper intracellular pH [84] and releases cellular contents in the surrounding environment [85]. It was also suggested that plasma species break down hydrogen, sulphide and peptide bonds of the proteins; leading to changes in protein structure and dramatically decrease of enzyme activity [86].

The inactivation of biological agents promoted by plasma may also result from deconstruction of the microorganism genetic material (DNA) by UV radiation produced with plasma and erosion of the microorganisms through intrinsic photodesorption. The photon-induced desorption results from the damage of chemical connections in the microorganism after being exposed to UV radiation, allowing its atoms to form volatile compounds [87,88]. However, the role of UV radiation in atmospheric-pressure plasma sterilization remains controversial [89]. According to some authors [88] even when no significant UV emission is present with low-temperature atmospheric-pressure plasma, the synergy of other species such as radicals and charged particles still plays a dominant role in sterilization.

Considering that plasma is usually produced by low-toxicity gases and its activity involves a mixture of products that decay within a few seconds, this approach has been suggested as environmentally friendly with no harmful residues [81]. Thus, production of stable plasma at atmospheric pressure has attracted attention for treating living human cells and tissues without thermal damage [81]. However, studies of the biological safety of plasma are limited [81], particularly on oral mucosa [81]. In contrast to conventional therapy, literature suggests that a great benefit on using plasma is that antimicrobial resistance is less likely to occur because of its multiple modes of action and diversity of active agents [18,86].

The current results showed identification of the *L. casei* group in all groups, however it did not show significant difference, which was previously demonstrated by a study that verified these bacteria as dominant in carie lesions in adults [90]. *Lactobacillus spp*. have the ability to produce organic acids, promoting low levels of pH and being responsible for the decalcification of the dentinal matrix [91,114], which is a common situation in carie lesions. Moreover, *Lactobacilli* have shown robust association with more advanced stages of caries in many studies [91,92,93,94,95,96,97] and have also been implicated in the initial stages of pulp infection [98], indicating that they present a pathogenic potential and play a crucial role in caries progression.

In the in vitro study by Rutger (2014) [114] in which a microbiological analysis was performed after treatment with plasma, plasma treatment of the agar plates caused complete inhibition of the growth of *E. coli* and *L. casei* in irradiated surface areas. In this investigation it was demonstrated that a plasma is suitable for substantially reducing oral microorganisms on agar plates or adherent to dentin slices, as well as the bacterium *E. coli*. These results confirm previously published data on the efficacy of cold plasma jets for killing and removing of planktonic or adherent micro-organisms [100,35,101]. Parts of agar plates contaminated with a density of 6 log10 CFU were nearly completely disinfected by plasma-jet treatment in the present study. Disinfection on directly irradiated areas was achieved at the shortest treatment time for *E. coli*, *L. casei* and *C. albicans*. For *S. mutans*, however, longer treatment periods were necessary.

In this study, the presence of *S. gordonii* was not significantly associated with treatment groups. The data is not consistent with those reported by Peterson et al. [109] in a dental plaque microbiome study in which *S. gordonii* was associated with caries and also with a metagenomic study that detected abundance of this species in individuals with caries [110]. The role of *S. gordonii* in dental biofilm is still undefined [111]. Despite being considered as a pioneer for dental plaque formation and associated with health [112], an in vitro study showed that these bacteria were able to increase their acid tolerance and acidogenicity when exposed to an acidic environment [113]. However, the contribution of *S. gordonii* on the dental biofilm remains unclear and deserves further investigation.

Another potential cariogenic pathogen recently identified is *Bifidobacterium dentium* [102]. It is closely related to gut commensal bifidobacteria, but it has acquired genes for survival in dental plaque at low pH, and does not colonize the edentulous mouth [103]. These various discoveries widen the view of the causative agents of dental caries past the *mutans streptococci*. In this study there was a statistical difference between the treatments, with treatment with plasma in relation to identification of *Bifidobacterium spp*. Low proportions of Bifidobacterium spp. were detected in dentine lesions in the NEVES study [14], which is in accordance with a previous study on adult biofilm lesions [92]. Bifidobacteria have been detected in dentine carious lesions [92, 61,94,95], suggesting that these bacteria may be implicated in dental caries progression [103], since these species are acidogenic and aciduric and also known to produce lactate [103,104]. Additionally, another study showed spatial distribution of bacterial taxa *in vivo* with confocal microscopy, showing a bacterial invasion into the dentinal tubules of *Bifidobacterium* inside cavitated caries lesions [105].

The results demonstrate that treatment of biofilms with reduced plasma, such as concentrations of *Streptococcus mutans*, *Lactobacillus acidophillus*, *Streptococcus mitis, Bifidibacterium* group and *Actinomyces naeslundis* compared to other treatment groups. This study provided results for a better understanding of the differences in microbiots after different antimicrobial treatments on biofilms. Further understanding and standardization are required to control microbial response and avoid possible cytotoxic effects of plasma. In addition, although no visibly damage was observed, direct plasma effects on tissues should be further investigated.

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Compliance with ethical standards The study protocol was approved by the ethics committee of the Federal University of Ceara, Brazil (COMEPE/UFC) (Protocol number 1.000.400). Verbal and

written consents were obtained of all subjects. Samples were taken only after obtaining the approval from the subjects.

Conflict of interest There are no potential conflicts of interest identified in this study.

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Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ans/ our national research committee and with the 1964 Helsink declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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4 CONCLUSÕES GERAIS

Inicialmente, o tratamento com luz de comprimento de onda de 400-690 nm pode ser uma abordagem terapêutica promissora para infecções por *C. albicans* relacionadas aos biofilmes, tais como a candidíase oral. Os resultados desse trabalho indicam que o tratamento duas vezes por dia dos biofilmes de *Cândida* com luz azul ou com luz vermelha, pode funcionar como um adjuvante a terapia antifúngica tópica.

Adicionalmente, a terapia utilizando um plasma de argônio de baixa temperatura foi eficaz em reduzir *S. mutans*, *L. acidophillus*, *S. mitis*, *Bifidobacterium* e *A. naeslundis* crescidas em biofilme oral maduro formado *in situ.* Com base na expressão gênica das bactérias presentes nas amostras, observou-se que o tratamento com o plasma foi mais eficaz para reduzir a viabilidade bacteriana do que a clorexidina, uma substância antimicrobiana padrão para a inibição da formação dos biofilmes.

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ANEXO

ANEXO A– PARECER DE APROVAÇÃO DA PESQUISA

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Atividade do plasma de baixa temperatura em biofilmes orais formados in situ
Pesquisador: Karla Shangela da Silva Alves
Área Temática:
Versão: 2
CAAE: 40975514.0.0000.5054
Instituição Proponente: UNIVERSIDADE FEDERAL DO CEARA
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.000.400
Data da Relatoria: 26/03/2015

Apresentação do Projeto:
Projeto de doutorado de Karla Shangela da Silva Alves sobre a utilizacao do plasma de baixa temperatura (PBT)na terapia anti-placa e anti-carie, ja que esta tecnologia provoca destruicao da matriz extracelular de bacterias e, estudos preliminares demonstraram a sua eficacia sobre biofilme oral maduro. Serao selecionados vinte voluntarios (estudantes do curso de Odontologia da FFOE-UFC) que receberao um dispositivo palatino intra-oral fabricado com metal, esmalte e tela para retencao de bacterias e formacao de biofilme. Apos 7(sete) dias, estes dispositivos serao removidos e tratados, em laboratorio, com terapia PBT. Cada amostra tera uma area irradiada e uma outra nao, esta ultima, considerado o controle. Apos o tratamento, os especimes serao analisados quanto a viabilidade bacteriana, propriedades bioquimicas da matriz do biofilme, determinacao das possiveis alteracoes da superficie do esmalte e caracterizacao molecular da populacao microbiana dos biofilmes tratados pela tecnica do PCR-DGGE. A Analise de Variancia e o teste Tukey serao utilizados tendo o nivel de significancia estabelecido em 5%.

Objetivo da Pesquisa:
Objetivo Primário:
Elucidar e aperfeiçoar uma abordagem única para um agente anti-placa, usando os efeitos sinérgicos do PBT de (i) destruir a matriz extracelular do biofilme maduro e (ii) a ação antimicrobiana, entretanto sem prejudicar o esmalte.

Endereço: Rua Cel. Nunes de Melo, 1127
Bairro: Rodolfo Teófilo **CEP:** 60.430-270
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

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Objetivo Secundario:
Avaliar o efeito do PBT em biofilmes formados in situ sobre blocos de esmalte

Avaliação dos Riscos e Benefícios:

A pesquisa apresenta baixo risco pois os voluntarios poderao sentir desconforto semelhante ao causado pelos aparelhos ortodonticos e apresentar mau halito durante o periodo experimental, o que podera ser controlado com a adequada higiene bucal e a limpeza do dispositivo intra-oral.
Quanto aos beneficios, os voluntarios serao acompanhados e receberao kits de higiene bucal. Os resultados deste projeto poderao estabelecer um protocolo eficiente nautilizacao do PBT para tratar biofilmes intra-orais.

Comentários e Considerações sobre a Pesquisa:

A pesquisa tem merito cientifico pois tentara introduzir novas tecnologias para a prevencao e o tratamento da carie dentaria em humanos. A utilizacao de um biofilme dentario real possibilitara dados consistentes sobre a terapia de PBT.

Considerações sobre os Termos de apresentação obrigatória:

A pesquisadora apresentou ao comite de etica: projeto de pesquisa, folha de rosto devidamente preenchida e assinada pelo chefe do DOR, declaracao de concordancia, autorizacao do laboratorio da PPGO, carta de encaminhamento, cronograma atualizado, orcamento, curriculo da pesqusadora principal e TCLE.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências éticas nem metodológicas.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

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UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

Continuação do Parecer: 1.000.400

FORTALEZA, 26 de Março de 2015

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
(Coordenador)

Endereço: Rua Cel. Nunes de Melo, 1127
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Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

ANEXO B– NORMAS PARA SUBMISSÃO DE ARTIGOS REVISTA A

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Title Page

The title page should include:

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- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, and telephone number(s) of the corresponding author
- If available, the 16-digit ORCID of the author(s)

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Purpose (stating the main purposes and research question)
- Methods
- Results
- Conclusions

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

Manuscripts should be submitted in Word.

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- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 182 kB)

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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

SCIENTIFIC STYLE

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Units and abbreviations

- Please adhere to internationally agreed standards such as those adopted by the commission of the International Union of Pure and Applied Physics (IUPAP) or defined by the International Organization of Standardization (ISO). Metric SI units should be used throughout except where non-SI units are more common [e.g. litre (l) for volume].
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- When drugs are mentioned, the international (generic) name should be used. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and methods.

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Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

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  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

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- Article by DOI

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- Book chapter

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- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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ARTWORK AND ILLUSTRATIONS GUIDELINES

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- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
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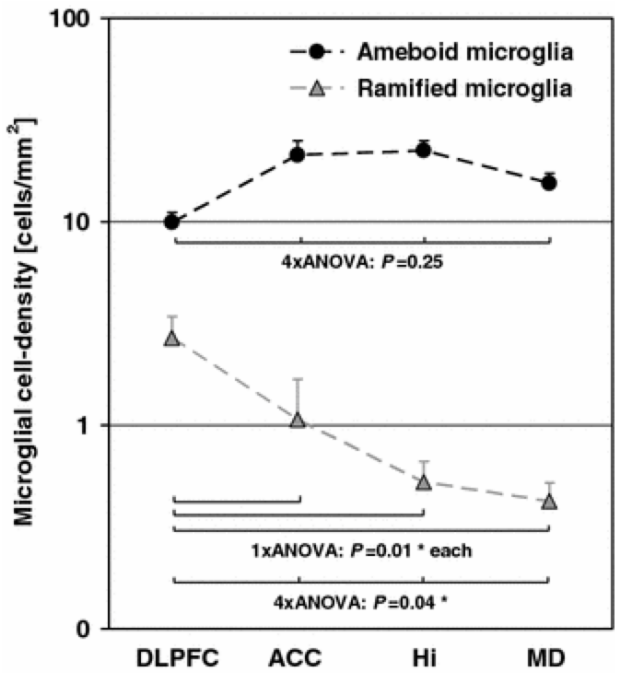

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

- All lines should be at least 0.1 mm (0.3 pt) wide.
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- Vector graphics containing fonts must have the fonts embedded in the files.

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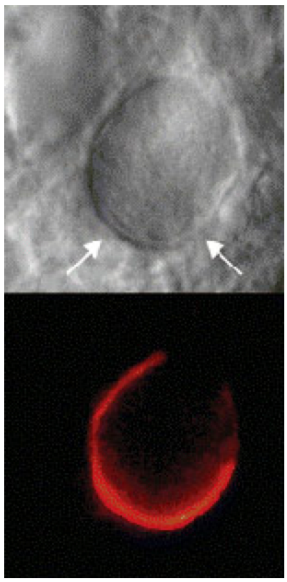
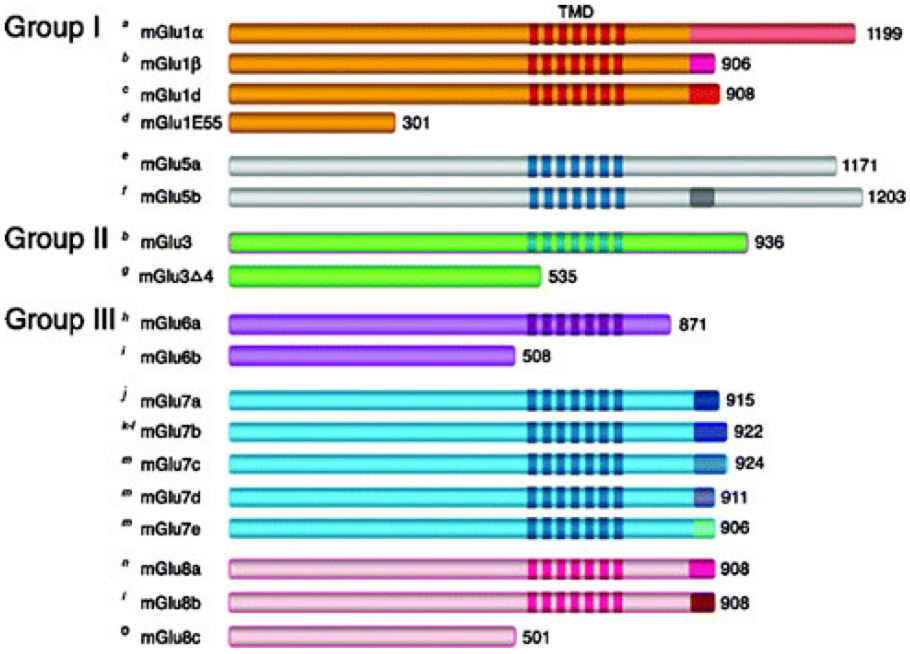


Combination Art



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- Research involving Human Participants and/or Animals
- Informed consent

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If you have any questions please contact:

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Department of Parodontology and Conservative Dentistry

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66421 Homburg/Saar

Germany

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Reference citations in the text should be identified by numbers in square brackets. Some examples:

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2. This result was later contradicted by Becker and Seligman [5].
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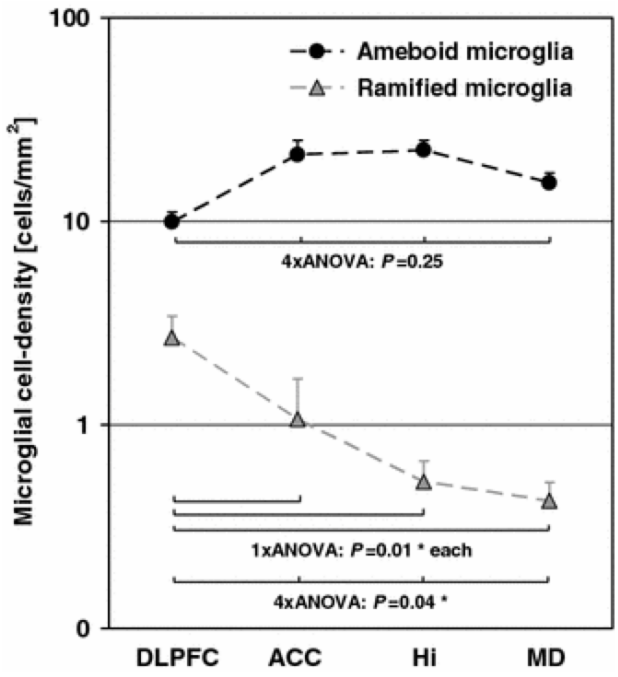

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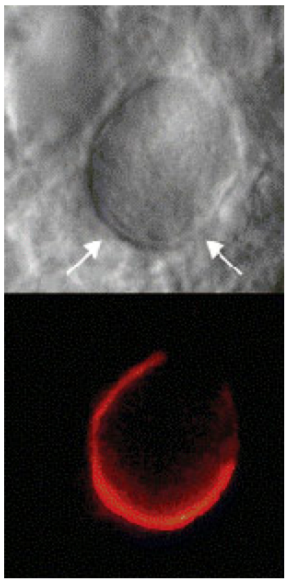
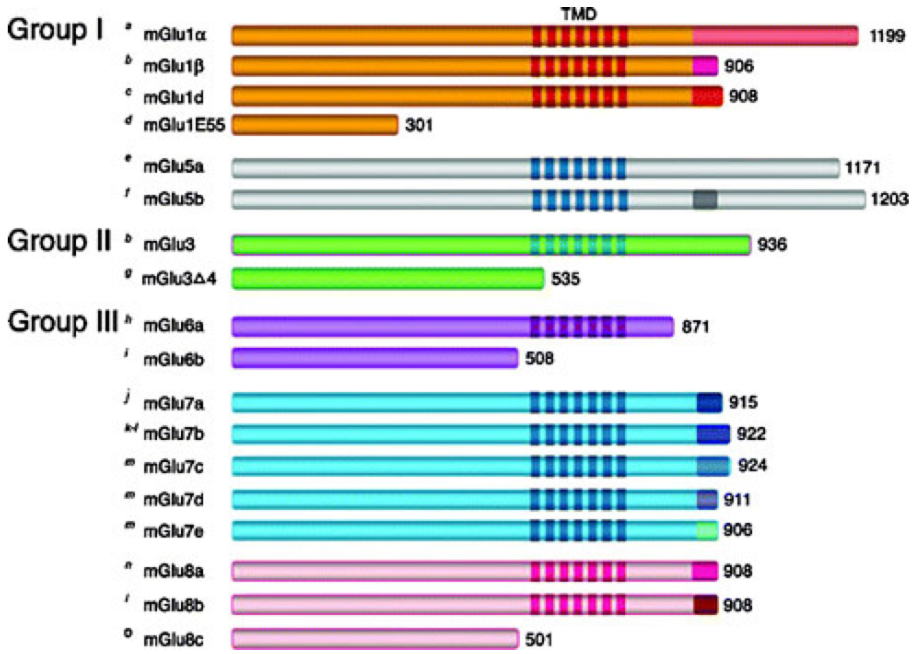


Combination Art



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