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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

BRUNO ROCHA DA SILVA

ATIVIDADE ANTIMICROBIANA E ANTIBIOFILME DO PEPTÍDEO KR-12 E SEU ANÁLOGO [W^7]*KR12*-KAEK CONTRA BACTÉRIAS RELACIONADAS À PATOLOGIAS ORAIS

FORTALEZA

2016

UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE MEDICINA
DEPARTAMENTO DE PATOLOGIA E MEDICINA LEGAL
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Aprovada em: ____/____/_____.

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“Olha aí! Ai, o meu guri, olha aí! Olha aí! É o meu guri e ele chega...” (Chico Buarque)

RESUMO

A microbiota oral é rica em diferentes espécies de bactérias, fungos e vírus. Geralmente, tais microorganismos coexistem em um ambiente de equilíbrio e benefício mútuo. Contudo, quando desordens de cunho ambiental, individual ou microbiológico acontecem, tal microbiota pode ser alterada causando a aparição de doenças como a cárie, a doença periodontal e lesões endodônticas. Atualmente, um grupo de moléculas bioativas tem despertado a atenção dos pesquisadores devido seu espectro de ação e biocompatibilidade, estes são os peptídeos antimicrobianos (PAMs). Dentre as diversas variedades de peptídeos, os relacionados ao sistema imune humano, como o KR-12 são os mais amplamente estudados, servindo como base para o desenvolvimento de novos peptídeos sintéticos com maior atividade biológica e biocompatibilidade. Dessa forma, o objetivo do presente trabalho foi avaliar*, in vitro*, o potencial antimicrobiano e antibiofilme do peptídeo sintético [W^7]KR12-KAEK sobre bactérias relacionadas ao desenvolvimento de patologias orais. Para se atingir o objetivo proposto, realizaram-se ensaios antibacterianos em células planctônicas e biofilmes, além de análise das atividades por microscopia eletrônica de varredura. Para os ensaios realizados, foram utilizadas as espécies *S. sanguinis* ATCC 10556, *S. sobrinus* ATCC 6715, *S. oralis* ATCC 10557, *S. salivarius* ATCC 7073, *S. parasanguinis* ATCC 903, *S. mutans* ATCC 25175, *S. mutans* UA 159, *S. mutans* UA 130, *E. faecalis* ATCC 10100 e *E. faecalis* ATCC 19433 na concentração $2x10^6$ µg/mL. Para análise de atividade antimicrobiana, as cepas bacterianas foram submetidas à técnica de microdiluição em placas de poliestireno para realização dos ensaios de concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM). Já para os ensaios de atividade antibiofilme, foram analisadas a quantificação de biomassa por cristal violeta e contagem de unidades formadoras tanto para o processo de inibição de biofilmes como para os testes em biofilmes pré-formados, além do uso de microscopia eletrônica de varredura (MEV) para análise morfológica de biofilmes pré-formados. Em relação à atividade contra bactérias planctônicas, o peptídeo sintético testado apresentou efeito antibacteriano significativo para todas as cepas testadas, com valores de CIM variando entre 7,8 e 31,25 µg/mL e valores de CBM entre 15,6 e 62,5 µg/mL. Para os ensaios de biofilmes, [W^7]KR12-KAEK mostrou uma redução na biomassa e número de UFC's para todas as bactérias testadas. Em relação ao ensaio de contagem de células viáveis dessas cepas, o peptídeo teste ocasionou uma considerável redução na quantidade de células nas estruturas de biofilme, mesmo nos ensaios de biofilmes maduros. Pela visualização por MEV, notou-se que na concentração de 500 µg/mL, o peptídeo ocasionou alterações morfológicas na superfície bacteriana de *S. mutans* UA 130 e *E. faecalis* ATCC 19433 (únicas cepas utilizadas para essa análise) e reduziu o número de células incrustadas no biofilme. Por fim, concluiu-se que o peptídeo [W^7]KR12-KAEK possui efetiva atividade antimicrobiana e antibiofilme contra as cepas avaliadas e é um potencial insumo biotecnológico para incorporação à produtos de uso odontológico no tratamento de patologias orais relacionadas a bactérias do gênero *Streptococcus* e *Enterococcus*.

Palavras-chave: Peptídeo Antimicrobiano, Agente Antimicrobiano, Carie Dental, *Streptococcus*, *Enterococcus*.

ABSTRACT

The oral microbiota is rich in different species of bacteria, fungi and viruses. Generally, these microorganisms coexist in a balanced environment for mutual benefit. However, when environmental, individual or microbiological factors cause disorders, such microbiota can be changed causing the appearance of diseases such as caries, periodontal disease and endodontic lesions. Currently, a group of bioactive molecules has attracted the attention of researchers because of its spectrum of action and biocompatibility, these are the antimicrobial peptides (AMPs). Among the several varieties of peptides, the ones related to the human immune system, such as KR-12, are the most widely studied, serving as a basis for the development of new synthetic peptide with greater biological activity and biocompatibility. Thus, the objective of this study was to evaluate the antimicrobial and antibiofilm potential of the synthetic peptide [W^7]KR12-KAEK on bacteria related to the development of oral diseases. To achieve this purpose, antibacterial assays were developed on planktonic cells and biofilms. Moreover, the peptide effect on preformed biofilms were analysed by scanning electron microscopy. For the tests performed, the species used were *S. sanguinis* ATCC 10556, *S. sobrinus* ATCC 6715, *S. oralis* ATCC 10557, *S. salivarius* ATCC 7073, *S. parasanguinis* ATCC 903, *S. mutans* ATCC 25175, *S. mutans* UA 159, *S. mutans* UA 130, *E. faecalis* ATCC 10100 and *E. faecalis* ATCC 19433. All strains were standardized for the cell concentration of $2x10^6$ CFU/ml. For antimicrobial analysis, bacterial strains were subjected to the technique of microdilution in microtiter plates to achieve the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For antibiofilm activity assays, were analyzed the biomass quantification by crystal violet and the counting of colony forming units on developing biofilms and preformed biofilms. The use of scanning electron microscopy (SEM) for morphological analysis was performed only for preformed biofilms. Regarding the activity against planktonic bacteria, synthetic peptide tested showed significant antibacterial effect for all strains tested, with MIC values ranging between 7.8 and 31.25 µg/mL and MBC values between 15.6 and 62.5 µg/ml. For biofilms tests, [W^7]KR12-KAEK showed a reduction in biomass and CFU's number for all tested bacteria, even in mature biofilm assays. The visualization by SEM showed that, at the concentration of 500 µg/ml, the peptide caused a morphological change in the bacterial surface of *S. mutans* UA130 and *E. faecalis* ATCC19433 (single strains used for this analysis) and reduced the number of embedded cells in the biofilm. Finally, it was concluded that the peptide [W^7]KR12-KAEK has effective antimicrobial and antibiofilm activity against all strains evaluated. The peptide has a biotechnological potential for incorporation into dental use products to treat oral disorders related to bacteria of the genus *Streptococcu*s and *Enterococcus*.

Keywords: Antimicrobial Peptide, Antimicrobial Agent, Dental Caries, *Streptococcus*, *Enterococcus.*

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LISTA DE ABREVIATURAS E SIGLAS

ABC	ATP binding cassete
AMP	Antimicrobial Peptide
ATCC	American Type Culture Collection
ATP	Adenosina Trifosfato
BHI	Brain Heart Infusion
CD	Cirlular Dicroism
CFU	Colony Forming Units
cm	Centímetro
CPO-D	Índice de Dentes Cariados, Perdidos e Obturados
DNA	Ácido Desoxiribonucléico
EDTA	Ácido Etilenodiamino Tetra-Acético
EPS	Substância Extracelular Polimérica
GTF	Glicosiltransferase
Ig	Imunoglobulina
IL	Interleucina
kDA	Kilodalton
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
ml	mililitro
mmol	Milimolar
nm	Nanômetro
OD	Densidade ótica
OMS	Organização Mundial de Saúde
OPG	Osteoprotegerina
PA	Pró-Análise
PAM	Peptídeo Antimicrobiano
PBS	Phosphate buffered saline
PG	Prostaglandina
pH	Potencial Hidrogeniônico
PMN	Polimorfonucleares
PTS	Sistema Fosfotransferase

RANK	Receptor ativador do fator nuclear kappa
RNA	Ácido Ribonucléico
SM	*Streptococcus mutans*
TFA	Terapia Fotodinâmica Antimicrobiana
TNF	Fator de Necrose Tumoral
UV	Ultravioleta
µg	micrograma
µl	microlitro
µM	microMolar

LISTA DE SÍMBOLOS

%	Porcentagem
°C	Grau Celsios
®	Marca registrada
<	menor que
A	Alanina
E	Glutamato
I	Isoleucina
K	Lisina
L	Leucina
R	Arginina
V	Valina
W	Triptofano
Y	Tirosina
α	alfa
β	beta

SUMÁRIO

Capítulo I – Introdução e Objetivos

I.1 INTRODUÇÃO

O conjunto de microorganismos encontrados na cavidade oral humana tem sido denominado de microflora oral, microbiota oral ou, mais recentemente, como microbioma oral. O termo microbioma oral foi introduzido por Lederberg e Mccray (2001) com a finalidade de conceituar a "comunidade ecológica de microorganismos comensais, simbióticos e patogênicos que literalmente compartilham nossa cavidade oral e são fatores determinantes no processo saúde e doença".

Aproximadamente 280 espécies bacterianas da cavidade oral já foram isoladas, identificadas e nomeadas. Contudo, estima-se que o número de microorganismos que colonizam o ambiente oral seja muito maior, em torno de 500-700 espécies diferentes (PASTER *et al*., 2001). Tal predição se baseia nos métodos escassos de cultivo ainda presentes e aos diversos nichos existentes na cavidade oral, que permitem uma gama de condições de concentração de oxigênio, pH, osmolaridade e quantidade de nutrientes (AAS *et al*., 2005; XU *et al*., 2015).

Contudo, a grande maioria dos microorganismos colonizadores da cavidade bucal não é capaz de sobreviver de forma planctônica, ou seja, disperso em solução isoladamente. Bactérias nessas condições seriam facilmente arrastadas da cavidade bucal por meio do fluxo salivar e pelo processo mastigatório. As bactérias da cavidade oral, bem como de outros sítios do corpo humano, se adequaram a outro estilo de vida em comunidades complexas bem orquestradas e organizadas entre si, denominadas de biofilmes (DEWHIRST *et al*., 2010; MARSH, 2010).

Os biofilmes são denominados como comunidades bacterianas uni ou multiespécies nas quais os microorganismos que a compõem são envoltos por uma matriz extracelular polimérica produzida por eles mesmos. Essa comunidade pode ser aderida ou não a uma determinada superfície e é um dos principais mecanismos de virulência dos microorganismos orais (BJARNSHOLT *et al*., 2013) envolvidos no processo cariogênico e de formação de lesões endodônticas (MARSH, 2010; JHAJHARIA *et al*., 2015)

A cárie dentária é conceituada como a destruição localizada de tecidos duros dentários susceptíveis por produtos ácidos provenientes da fermentação bacteriana de carboidratos (SELWITZ; ISMAIL; PITTS, 2007). É considerada como uma das principais doenças infecciosas preveníveis em pacientes pediátricos; muito embora os indivíduos possam ser acometidos por tal doença ao longo de toda a vida (HOUSHMAND *et al*., 2012; SUNI *et al*., 2013).

O principal fator etiológico relacionado com o desenvolvimento da cárie dentária é a colonização de microorganismos patogênicos sobre as superfícies orais. Tais microorganismos variam de acordo com o grau de desenvolvimento da lesão e fatores do hospedeiro. Contudo, a habilidade desses colonizadores em se desenvolverem em biofilmes é um dos fatores de virulência mais importantes e, consequentemente, um dos mais estudados (KOLENBRANDER *et al*., 2010).

As bactérias do gênero *Streptococcus*, em particular as espécies do grupo mutans, são conhecidas por participarem ativamente do processo de desmineralização dentário que culmina na cárie dental. Isso se deve, dentre outros fatores, a sua elevada capacidade de fermentação dos carboidratos da dieta humana e eliminação de ácidos que causam a queda de pH localmente (TAKAHASHI; NYVAD, 2011).

A cárie é a maior causa de perdas de unidades dentárias e dor orofacial, apesar de poder ser revertida em seus estágios iniciais. Contudo, tal patologia não é auto limitante, o que, sem os cuidados adequados, pode levar a sua progressão até o acometimento pulpar ou a completa destruição do elemento dentário (BRETZ; ROSA, 2011).

O acometimento endodôntico é uma sequela comum do avanço das lesões cariosas em todo o mundo (PAK; FAYAZI; WHITE, 2012). A medida que a desmineralização dos tecidos duros dentais avança, a cavidade progride em direção cervical e pode atingir a polpa dentária, descarregando sobre ela uma infinidade de microorganismos (ZEHNDER; BELIBASAKIS, 2015; SIQUEIRA *et al*., 2014).

Quando da colonização da cavidade pulpar, o tecido mole inicia um processo inflamatório inicial, denominado de pulpite, que, em estágios iniciais é reversível, mas que em um curto espaço de tempo culmina na necrose do tecido pulpar e completa colonização microbiana pelos canais e túbulos dentinarios radiculares. Não dificilmente, os microorganismos colonizam a região periapical do dente acometido e inicia um processo inflamatório reabsortivo em tecido ósseo, causando os chamados abscessos odontogênicos (ZEHNDER; BELIBASAKIS, 2015; SIQUEIRA *et al*., 2014).

Muito embora geralmente as infecções endodônticas tenham origem das lesões de cárie, a microbiota colonizadora dos canais radiculares é extremamente diferente. Vários microorganismos dos gêneros *Prevotella, Porphyromonas, Tannerella, Fusobacterium* e *Treponema* estão diretamente relacionados com as infecções primárias nos canais radiculares (NARAYANAN; VAISHNAVI, 2010). Porém, uma das espécies mais estudadas na atualidade devido seu envolvimento com lesões apicais persistentes e falhas no tratamento é a *Enterococcus faecalis* (LOVE, 2001; ZHANG; DU; PENG, 2015)

O *E. faecalis* é uma bactéria notoriamente resistente às condições ambientais e agentes químicos agressores. Tais características se devem tanto aos seus mecanismos genéticos e celulares de resistência, como também a sua facilidade de se aglomerar em biofilmes ao longo dos canais radiculares e até mesmo no interior dos túbulos dentinarios (JHAJHARIA *et al*., 2015).

Devido a importância dos diversos microorganismos orais no processo de etiopatogênese das principais doenças orais, diversos estudos têm sido realizados com vista ao controle do microbioma oral e, consequentemente, ao combate contra o desenvolvimento de biofilmes orais (CARNEIRO *et al*., 2010; SÁ *et al*., 2012; WANG *et al*., 2012).

Neste contexto, um grupo de moléculas com características peculiares tem sido foco de pesquisas no campo da Microbiologia, os peptídeos antimicrobianos (PAMs). Os PAMs são um grupo heterogêneo de moléculas encontrados em diversos organismos, de insetos e anfíbios a mamíferos, além de possuírem ampla atividade antibacteriana e antifúngica (DA SILVA *et al*., 2012).

Tais moléculas têm recebido bastante atenção, pois elas agem na membrana celular do patógeno, estrutura essa que não é um alvo quiral específico. Dessa forma, bactérias e fungos têm dificuldade em superar os efeitos deletérios induzidos pelos PAMs (CRUSCA *et al*., 2011).

Dentre os diversos peptídeos conhecidos, os derivados do sistema imune inato humano são alguns dos mais estudados, dos quais destaca-se a Catelicidina LL-37. Este peptídeo é uma molécula anfipática, catiônica com 37 resíduos de aminoácidos de comprimento. Possui uma atividade marcante contra microorganismos Gram-negativos, muito embora possua um tamanho consideravelmente longo para viabilizar uma droga com finalidade terapêutica (DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006; JOHANSSON *et al*., 1998).

Um dos derivados bioativos do LL-37 é o peptídeo KR-12 que compreende os resíduos 18-29 do LL-37 e é um dos menores peptídeos conhecidos com atividade antimicrobiana. Devido sua atividade antibacteriana relevante e seu pequeno tamanho, o KR-12 se tornou um modelo extremamente viável para a construção de peptídeos sintéticos modificados (MISHRA *et al*., 2013).

I.2 OBJETIVOS

I.2.1 Objetivo Geral

Avaliar o potencial antimicrobiano e antibiofilme*, in vitro*, do peptídeo sintético [W^7]KR12-KAEK sobre bactérias relacionadas ao desenvolvimento de patologias orais.

I.2.2 Objetivos Específicos

- Descrever a síntese de um peptídeo inédito utilizando-se o peptídeo nativo KR-12 como modelo base;
- Caracterizar físico-queímicamente o peptídeo sintético [W^7]KR12-KAEK;
- Avaliar a atividade antimicrobiana do peptídeo [W^7]KR12-KAEK sobre o crescimento planctônico das cepas bacterianas *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 6715, *Streptococcus oralis* ATCC 10557, *Streptococcus salivarius* ATCC 7073, *Streptococcus parasanguinis* ATCC 903, *Streptococcus mutans* ATCC 25175, *Streptococcus mutans* UA 159, *Streptococcus mutans* UA 130, *Enterococcus faecalis* ATCC 10100 e *Enterococcus faecalis* ATCC 19433;
- Analisar a atividade do peptídeo [W^7]KR12-KAEK de interferir na formação inicial de biofilmes referentes às cepas supracitadas;
- Verificar a capacidade antibiofilme do peptídeo [W^7]KR12-KAEK sobre biofilmes pré-formados referentes às cepas supracitadas;
- Apontar as alterações morfológicas e estruturais, por microscopia eletrônica de varredura, de biofilme pré-formado das espécies *Streptococcus mutans* UA 130 e *Enterococcus faecalis* ATCC 19433 após contato com o peptídeo [W^7]KR12-KAEK.

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Capítulo II – Revisão de literatura

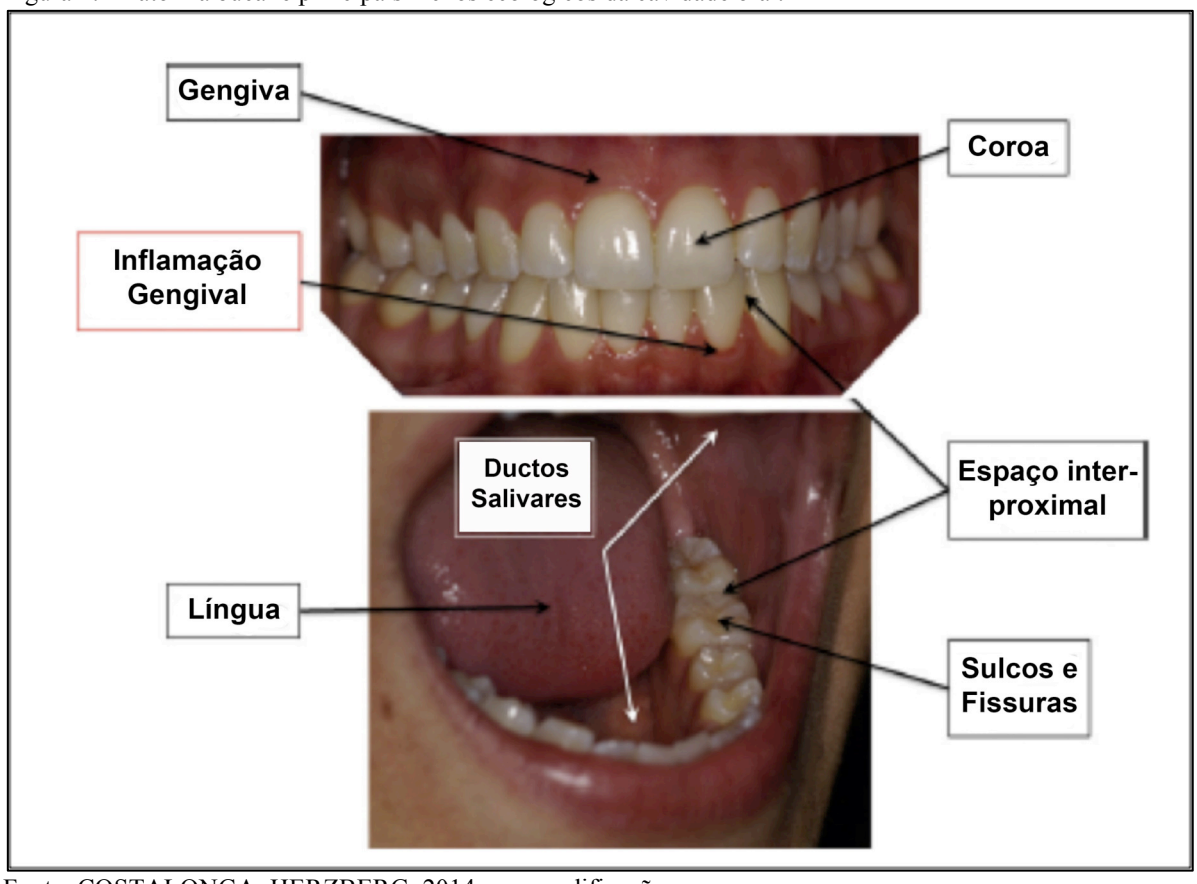
II.1 MICROBIOMA ORAL

II.1.1 Aspectos Gerais

A região oral e orofaringe são ambientes altamente propícios ao desenvolvimento microbiano. Além de possuírem temperatura ideal para crescimento e uma fonte inesgotável de alimentação, estas áreas possuem uma variedade de ambientes específicos e com condições propícias ao crescimento de diversas espécies bacterianas e fúngicas (WADE, 2013).

Dentre os diversos nichos de crescimento presentes na cavidade oral, os mais importantes e mais frequentemente estudados são as superfícies e sulcos gengivais, coroa dentária, sulcos e fissuras oclusais, espaços interproximais e língua (FIGURA 1) (ZAURA *et al*., 2009).

Figura 1: Anatomia bucal e principais nichos ecológicos da cavidade oral.



Fonte: COSTALONGA; HERZBERG, 2014 com modificações.

A superfície coronária, gengival e lingual geralmente possuem microbiomas semelhantes em virtude da sua localização anatômica e com fluxo intenso de alimento e oxigenação (SIQUEIRA; CUSTODIO; MCDONALD, 2012). Geralmente cocos e bacilos aeróbios Gram-positivos são os microorganismos mais prevalentes. Microbioma semelhante

também pode ser encontrada nas regiões de sulcos e fissuras oclusais, contudo, apesar dos grupos parecidos, a quantidade de bactérias com capacidade acidogênica é consideravelmente elevada nesses locais. *Streptococcus* do grupo mutans, além de *Lactobacillus* podem ser encontrados em abundância nessas regiões (KOLENBRANDER *et al*., 2010).

Já os sulcos gengivais e espaços interproximais possuem uma gama de microorganismos consideravelmente diferente. Devido à diminuição do gradiente de oxigênio nesses nichos, algumas outras bactérias como bacilos e espiroquetas anaeróbicas ou aeróbicas facultativas Gram-negativas são predominantes. Gêneros como *Treponema, Fusobacterium, Prevotella* e *Porphyromonas* são os mais comuns nesses ambientes (HAYASHI *et al*., 2010).

Muito embora a variedade de nichos ecológicos orais seja grande e, consequentemente, o número de microorganismos que os habitam seja maior ainda, pode-se pensar que tais seres vivem isoladamente em cada nicho. Contudo, hoje já é claro que o conjunto de seres vivos que constituem a microbioma oral vive harmonicamente e de forma ecológica, mantendo um equilíbrio entre todos os seres orquestrado pelos diversos fatores que podem influênciar seu crescimento. Devido às características de interrelação estabelecidas entre as espécies microbianas orais, atualmente o termo microbioma oral é o mais aceito em todo o mundo (COSTALONGA; HERSBERG, 2014).

II.1.2 Microbioma Oral

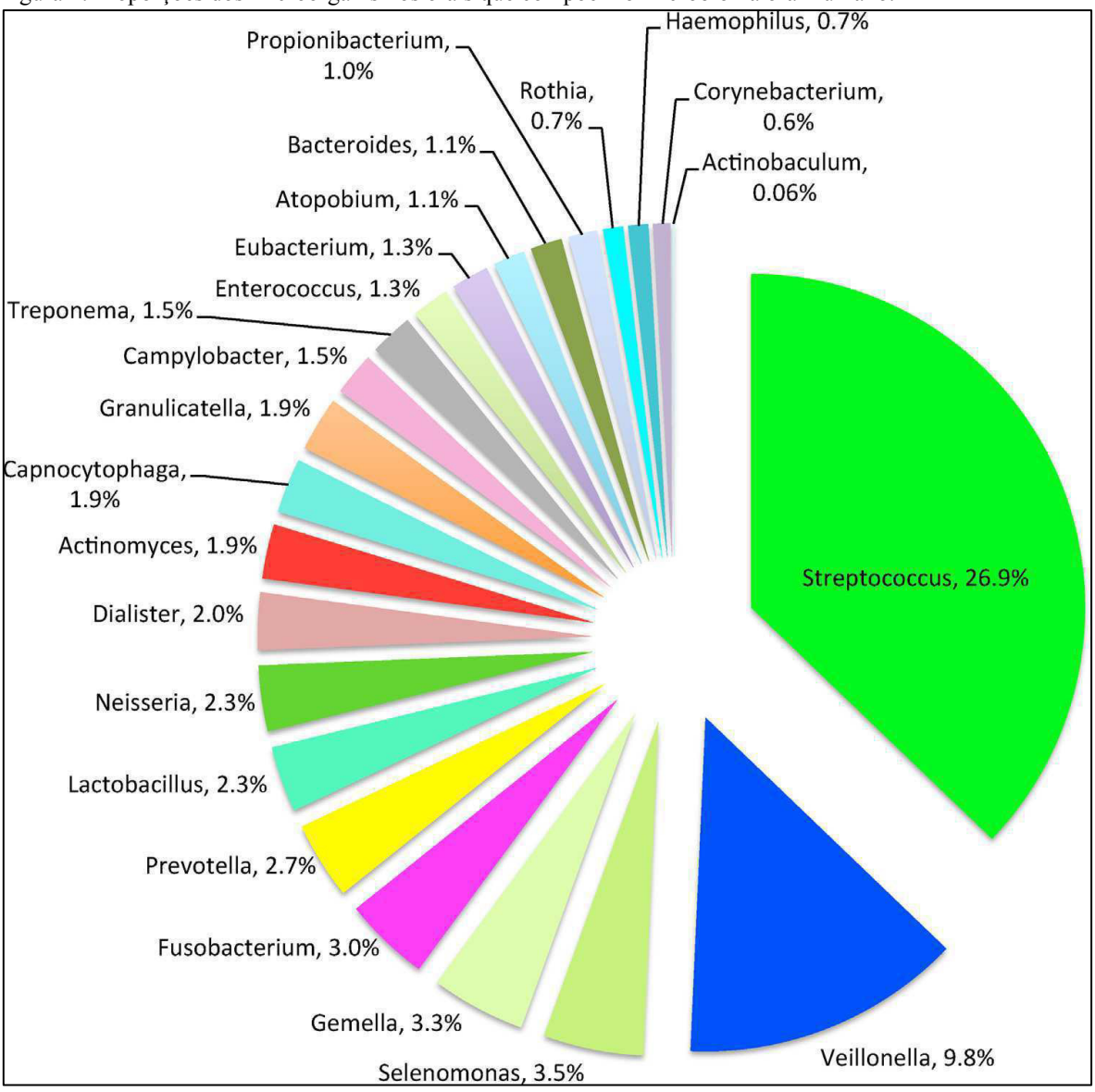
O termo Bioma é complexo e, por vezes, ainda não definido. Contudo, segundo Coutinho (2006), bioma é conceituado como um conjunto de ecossistemas devidamente equilibrados e harmoniosos em uma determinada área bem estabelecida. Muito embora tal conceito seja aplicado para regiões com vasta flora e fauna, quando aplica-se esse termo ao ambiente microscópico, é notável o quanto ele é útil e preciso (WADE, 2013).

O microbioma oral é composto por mais de 1000 espécies diferentes, entre já conhecidas e ainda desconhecidas, somente atrás do microbioma do cólon humano. Esse microbioma é composto por seres vivos dos mais diversos filos e gêneros, como vírus, bactérias, fungos e até mesmo protozoários (DEWHIRST *et al*., 2010). Além disso, o microbioma oral humano ainda engloba microorganismos comensais, simbióticos e patogênicos, em que a população dominante está diretamente relacionada aos fatores ambientais e do hospedeiro (FIGURA 2) (AAS *et al*., 2005; WADE, 2013).

O microbioma oral desempenha papéis importantes na manutenção da saúde oral e sistêmica do indivíduo. Bactérias comensais presentes na orofaringe são conhecidas por

serem essenciais ao desenvolvimento de estruturas anatômicas locais e para o desenvolvimento apropriado da imunidade local e sistêmica (HOOPER; LITTMAN; MACPHERSON, 2012; SHROFF; MESLIN; CEBRA, 1995).

Figura 2: Proporções dos microorganismos orais que compõem o microbioma oral humano.



Fonte: COSTALONGA; HERZBERG, 2014.

Somente a presença do microbioma na cavidade oral já é capaz de inibir a colonização por patógenos não naturais do ambiente. Introduzido inicialmente em 1994 por Vollaard e Clasener, o conceito de "resistência à colonização" ganhou ênfase nas últimas décadas através de estudos que comprovam que determinadas espécies nativas da boca impedem a adesão de certas espécies externas pela ocupação dos sítios de ligação na superfície oral. Além disso, tal efeito também pode ser exemplificado no uso crônico de

substâncias antimicrobianas que, por sua vez, desregulam o microbioma oral e permite que outros microorganismos oportunistas se desenvolvam causando doenças (SULLIVAN; EDLUND; NORD, 2001).

Algumas bactérias orais que estão associadas à condições saudáveis podem também ser antagonistas ao desenvolvimento de patógenos orais. Por exemplo, *Streptococcus salivarius* K12 produz uma toxina capaz de inibir o crescimento de espécies Gram-negativas que estão frequentemente associadas à doenças periodontais e halitose *in vitro*, e tem sido demonstrada com efeitos benéficos contra halitose *in vivo* (WESCOMBE *et al*., 2009).

Uma outra característica interessante do microbioma oral está relacionada com o seu metabolismo de nitrato e suas consequências com a saúde cardiovascular. Em torno de um quarto do nitrato ingerido retorna para a cavidade oral por meio de uma rota denominada de enterosalivar. Bactérias orais reduzem o nitrato a nitrito o qual é absorvido para as vias sanguíneas e convertido em óxido nítrico. Tal molécula é essencial para a saúde vascular pois ajuda a manter a integridade vascular e possui propriedades anti-hipertensivas (KAPIL *et al*., 2010; GOVONI *et al*., 2008; PETERSSON *et al*., 2009).

Grande parte dos microorganismos que colonizam a cavidade oral possuem mecanismos específicos e adaptados para a melhor sobrevivência em determinado nicho. Um dos recursos mais comuns e mais amplamente estudados na atualidade é a capacidade dos microorganismos orais se desenvolverem em biofilmes.

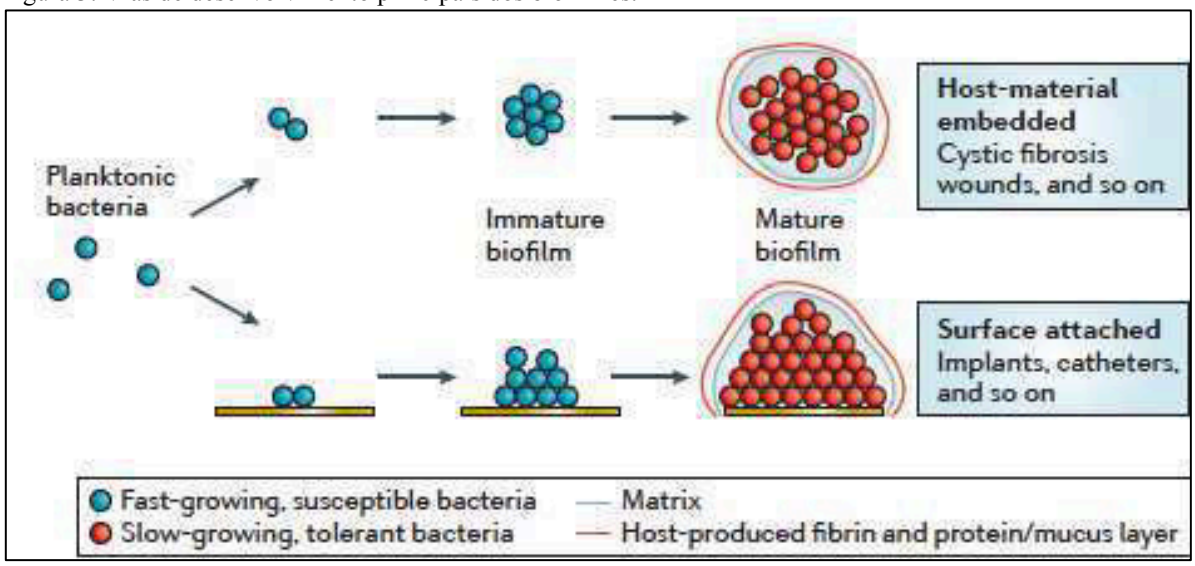
II.1.3 Biofilmes Orais

Os biofilmes são comunidades microbianas complexas estabelecidas em uma ampla variedade de superfícies que são geralmente associadas a uma matriz extracelular composta por vários tipos de biopolímeros (ABEE *et al*., 2010). Esse tipo de associação microbiana pode se desenvolver em diversas superfícies, sejam essas bióticas ou abióticas. Locais como tecidos vivos, dispositivos médicos e/ou industrias, sistemas de tubulação de água ou até mesmo ambientes marinhos podem se tornar propícios para o desenvolvimento de biofilmes. Na medicina, as superfícies, tais como dentes, catéteres, válvulas cardíacas e lentes de contato podem ser fortemente colonizadas (AKCALI *et al*., 2013; ONURDAĞ, *et al*., 2010; WILLCOX, 2013).

Contudo, com o avanço da tecnologia moderna, atualmente a visão de como os biofilmes são estruturados e formados avançou. Segundo Bjarnsholt e colaboradores, em 2013, os biofilmes não são obrigatoriamente desenvolvidos sobre uma superfície sólida ou

semi-sólida. Além da possibilidade de formação de biofilmes complexos em superfícies líquidas, agregados bacterianos envoltos em uma matriz polimérica extracelular podem ser encontrados em solução. Tais agregados apresentariam as mesmas características genéticas, estruturais, morfológicas e celulares dos biofilmes encontrados aderidos à superfícies (MULLER; SEIDLER; BEAUVAIS, 2011; CUELLAR-CRUZ *et al*., 2012) (FIGURA 3).

Figura 3: Vias de desenvolvimento principais dos biofilmes.



Fonte: BJARNSHOLT *et al*., 2013.

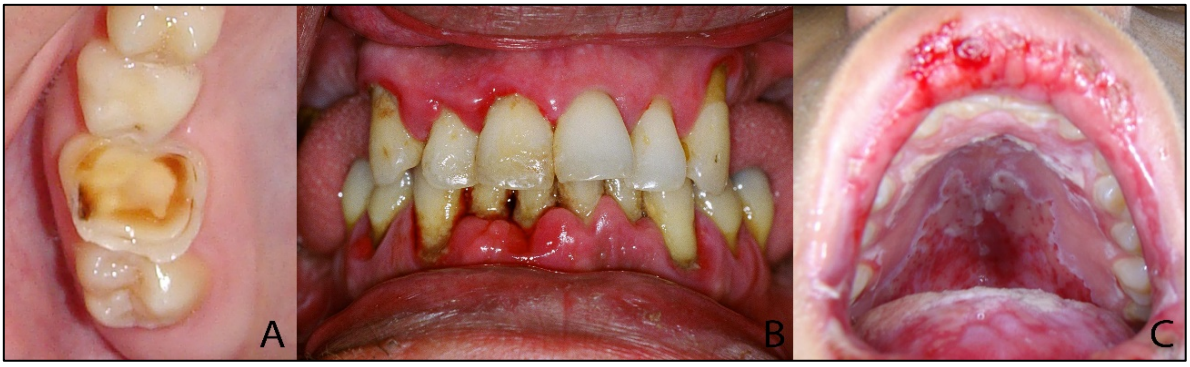
No caso dos biofilmes orais, não existem evidências claras da existência de biofilmes dispersos em líquido mucoso ou salivar. Contudo, a presença intensa e constante de fluidos na cavidade oral facilita a dispersão de microorganismos isolados ou até mesmo fragmentos de biofilmes previamente formados aderidos em um dos diversos nichos da cavidade oral (BJARNSHOLT *et al*., 2013).

O crescimento e a proliferação do microorganismo dentro de um biofilme fornece proteção contra a ação de antibióticos, proporcionando um mecanismo de defesa, diminuindo ou impedindo a penetração de diferentes agentes antimicrobianos através do biofilme (EPSTEIN *et al*., 2011; SOTO, 2013), e assim, tornam-se extremamente difíceis ou impossíveis de erradicar (ALHEDE *et al*, 2009; VAN GENNIP *et al*., 2009).

No caso dos biofilmes patogênicos orais, diversos grupos de pesquisa têm direcionado esforços para a melhor compreensão tanto dos seus aspectos de formação e desenvolvimento, quanto no seu metabolismo e composição (APATZIDOU, 2012; ZIJNGE *et al*., 2012). Tais estudos possuem, em geral, o objetivo final de desenvolver ferramentas

eficazes no controle da microbioma responsável por diversas doenças orais (cáries, periodontite, candidíase, doenças cardíacas e pulmonares) (Figura 4).

Figura 4: Lesões orais mais prevalentes com etiologia microbiana. A) Lesão de cárie extensa; B) Periodontite crônica severa; C) Candidíase eritematosa em paciente portador de imunodeficiência.



Fonte: Arquivo do autor.

II.1.4 Processo de Formação dos Biofilmes Orais

O processo de desenvolvimento dos biofilmes orais é complexo e segue uma cronologia bastante criteriosa. Em geral, todas as bactérias orais possuem mecanismos de aderência à superfícies sólidas recobertas por películas salivares, como os dentes, ou superfícies descamativas, como o tecido epitelial, ou, até mesmo, a bactérias que já estão previamente aderidas à superfície (DANNE; DRAMSI, 2012; KOLENBRANDER, 2011).

A aderência de células microbianas a outras bactérias imobilizadas sobre uma superfície é chamada de coadesão, enquanto que a ligação de bactérias ou microorganismos em suspensão é chamada de coagregação (KOLENBRANDER *et al*., 2010). Dessa forma, a construção do biofilme oral é realizada inicialmente por determinadas estirpes bacterianas que possuem a capacidade de se aderir à película adquirida no esmalte dentário. Tais bactérias são chamadas de colonizadores iniciais e seus principais representantes são os *Streptococcus oralis, Streptococcus sanguinis, Streptococcus parasanguinis* e *Streptococcus gordonii* para os biofilmes predominantemente cariogênicos e outras bactérias do gênero Actinomices para os biofilmes periodontopatogênicos. Tal relação dos colonizadores iniciais com o processo patológico é advinda da própria seletividade bacteriana no sistema de coadesão, contudo, tal estratégia não impede a participação cruzada desses colonizadores iniciais nos processos patológicos (BEIER; QUIVEY; BERGER, 2012; KOLENBRANDER *et al*., 2010).

Após a adesão inicial por parte dos colonizadores primários, o processo segue com a proliferação dessas células e o início da adesão de outras células com receptores de afinidade (adesinas e selectinas) que possuem a capacidade de se aderir seletivamente a outros

microorganismos (BADIHI-HAUSLICH *et al*., 2011). No processo cariogênico, o *Streptococcus mutans* e o *Streptococcus sobrinus* merecem uma atenção especial, enquanto que no processo periodontopatogênico se destacam o *Fusobacterium nucleatum* e a *Porphyromonas gingivalis* (KOLENBRANDER, 2011; KOLENBRANDER *et al*., 2010). Devido a importância crucial desses microganismos na sua sustentabilidade patogênica, vários estudos são voltados para a descoberta de novos métodos de controlá-los.

Apesar de não serem colonizadores iniciais do biofilme cariogênico, *Streptococcus mutans* e *Streptococcus sobrinus* são os principais componentes responsáveis pelo processo de formação da cavidade de cárie. Este fato se deve a capacidade tanto de produzir ácido que promove o processo de desmineralização dentária, como em sobreviver nos ambientes com pH ácido (BOWEN; KOO, 2011).

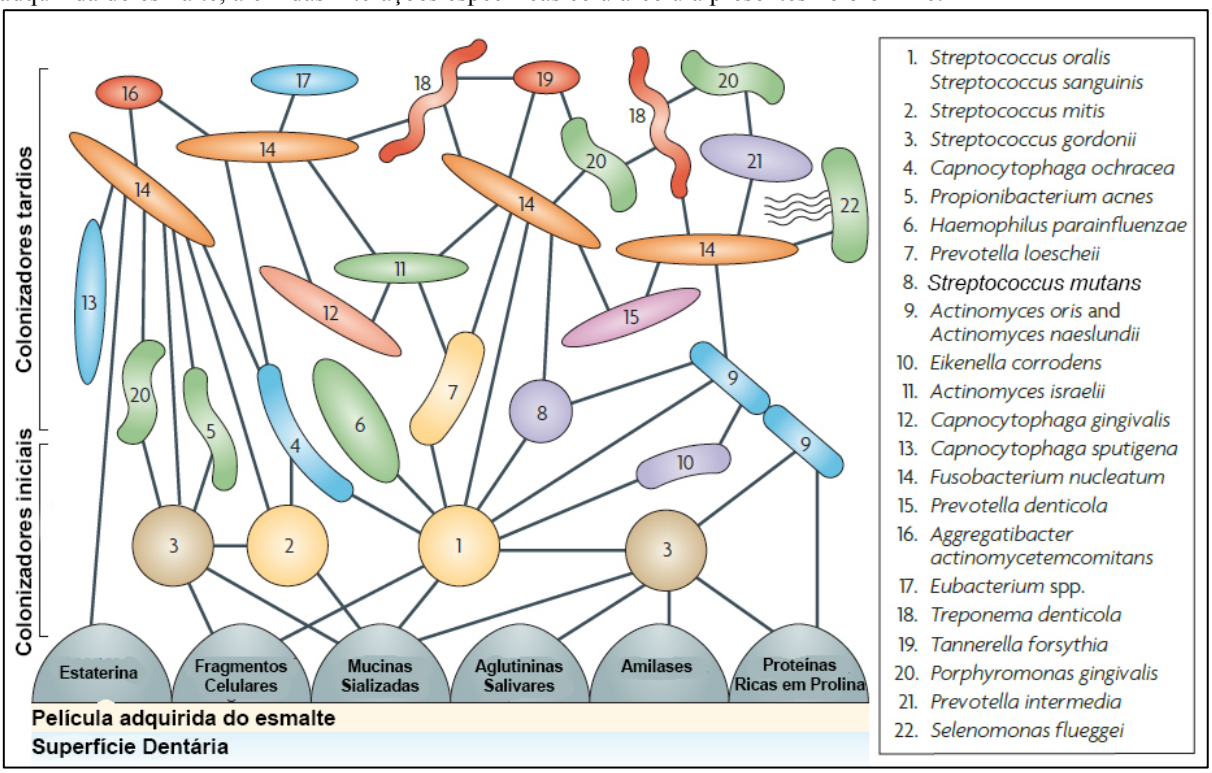
Já as bactérias periodontopatogênicas não possuem o potencial acidogênico, mas são capazes de formar biofilmes mais resistentes e secretar enzimas líticas que degradam o tecido periodontal (DEO; BHONGADE, 2010; TAKEUCHI *et al*., 2011). Além disso, a característica mais predominante das bactérias periodontopatogênicas é a sua capacidade de sobrevivência em anaerobiose ou aerobiose facultativa (TENORIO *et al*., 2011).

A medida que colonizadores secundários são gradativamente acumulados sobre o biofilme, unidades especializadas denominadas microcolônias começam a ser plenamente estabelecidas (KOLENBRANDER *et al*., 2010). Essas microcolônias são estruturas fundamentais para o funcionamento metabólico e organizacional do biofilme, como será abordado a seguir. Na figura abaixo, pode-se evidenciar um esquema organizacional didático referente à estruturação em biofilmes das bactérias que residem em ambiente oral (FIGURA 5).

II.1.5 Metabolismo do Biofilme

Para um completo desenvolvimento e maturação dos biofilmes tanto presentes em cavidade oral como na grande maioria dos ambientes, os substratos metabólicos precisam estar disponíveis para as células do biofilme, para assim possibilitar seu crescimento. Tais compostos encontram-se geralmente solubilizados em saliva pois são frequentemente frutos do processo digestivo do próprio hospedeiro (CCAHUANA-VÁSQUEZ; CURY, 2010; FIELD *et al*., 2010).

Figura 5: Modelo espaço temporal de colonização bacteriana em cavidade oral, no qual se evidencia a interação entre os receptores moleculares presentes na superfície bacteriana e as diversas moléculas presentes na película adquirida do esmalte, além das interações específicas célula-célula presentes no biofilme.



Fonte: KOLENBRANDER *et al*., 2010 com modificações.

Grande parte da diversidade metabólica de um biofilme pode ser explicada pelos diferentes solutos químicos presentes no ambiente. Biofilmes maduros possuem um gradiente de concentração de substratos e de produtos metabólicos (BABAUTA *et al*., 2012; KOLEY *et al*., 2011). Dessa forma, podem haver regiões no interior do biofilme em que o crescimento bacteriano e a atividade metabólica são inibidos ou retardados devido à limitação de substrato. Além disso, o acúmulo de resíduos ácidos no interior do biofilme poderiam reduzir o pH local e afetar diretamente as respostas fisiológicas e metabólicas de algumas bactérias (BABAUTA *et al*., 2012).

Quando as bactérias utilizam glicoproteínas como fonte de açúcar, estas lançam mão de exoglicosidases em combinação com um sistema de transporte de açúcar, além de enzimas glicolíticas específicas para cada carboidrato. Açúcares podem ser translocados para o citoplasma por vários sistemas de transporte (LIU *et al*., 2012). Um dos mais importantes é o sistema fosfotransferase (PTS), o qual possui um papel regulador fundamental no controle do metabolismo de carbono em bactérias, gram-positivas e negativas. PTS também controla outros sistemas de transporte, metabolismo e armazenagem de fontes de carbono e de nitrogénio. O sistema de transporte de açúcar regulado pela PTS é do tipo simporte prótons-

açúcar e Na^+-açúcar (AJDIC; CHEN, 2012; LIU *et al*., 2012). O objetivo final é uma utilização eficiente da oferta limitada de carbono e recursos energéticos.

A presença de sacarose permite a aderência de estreptococos do grupo mutans e consequentemente a colonização da superfície lisa do esmalte dental, favorecendo seu posterior acúmulo (AIRES *et al*., 2008). Esses microorganismos não só fermentam a sacarose como, a partir desta, sintetizam polissacarídeos extracelulares (KLEIN *et al*., 2012). Na polimerização desses açúcares participam as enzimas denominadas glicosiltransferases (Gtfs), para as glicanas, e frutosiltransferases (Ftfs), para as frutanas, que são essenciais para expressão da virulência (FELDMAN *et al*., 2009; YOUSEFI *et al*., 2012). Essas enzimas atuam na produção de glicanos solúveis e insolúveis em água que servem de reserva energética intracelular, fonte de carbono, e também como um aderente para facilitar a formação do biofilme (BANAS; BISWAS; ZHU, 2011).

II.1.6 Matriz Extracelular Polimérica

Biofilmes são formados principalmente por células microbianas e substâncias extracelulares poliméricas, mais conhecidas como exopolissacarideos ou simplesmente EPS. Essas substâncias podem ser responsáveis por 50% a 90% do carbono orgânico total do biofilme (KUBONIWA *et al*., 2012) e podem ser consideradas o principal componente da matriz do biofilme. O EPS, formado principalmente de polissacarídeos, pode variar segundo as propriedades químicas e físicas, como a solubilidade em água. Alguns desses carboidratos são neutros ou polianiônicos, como é o caso do EPS de bactérias gram-negativas, por conta da presença de ácidos urônicos, D-glucurônico, D-galacturônico, e outros (XIAO *et al*., 2012). No caso de algumas bactérias gram-positivas, tais como os estafilococos, a composição química do EPS pode ser bastante diferente, mas com características catiônicas. Muitas vezes, a composição e a quantidade de EPS variarão dependendo do tipo de microorganismos, da idade do biofilme e das diferentes condições ambientais em que os biofilmes são formados (KUBONIWA *et al*., 2012). Estes incluem diferentes níveis de oxigénio e de nitrogênio, de pH, de temperatura e de disponibilidade de nutrientes (AHIMOU *et al*., 2007).

Segundo Xiao e colaboradores (2012) o EPS pode ter um efeito significativo sobre a organização do biofilme. A composição dos polissacarídeos determina sua conformação primária. Por exemplo, muitos EPS possuem estruturas com o esqueleto principal formado por resíduos de hexoses com ligações β-1,3 ou β-1,4, com isso tendem a

possuir uma estrutura mais rígida e resistente, com pouca ou nenhuma solubilidade em água (XIAO *et al*., 2012).

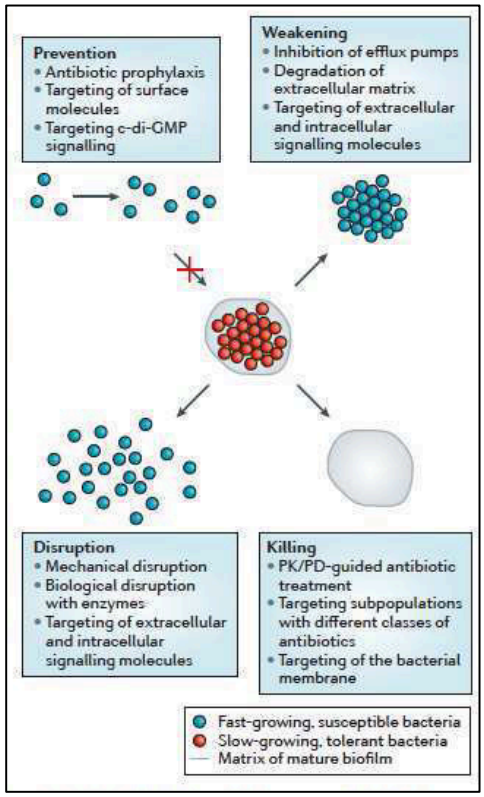
O crescimento de um biofilme é o resultado de um processo complexo que envolve o transporte de compostos orgânicos, moléculas inorgânicas e células microbianas, uma posterior adesão em uma superfície e finalmente, a formação de um consórcio bacteriano auxiliado pela produção de polímeros extracelulares (IRIE *et al*., 2012). Assim como a hidrofobicidade celular e a presença de fímbrias e de flagelos, o grau de produção de EPS é um dos principais fatores que influencia a taxa e o grau de adesão de células microbianas em diferentes superfícies, além de proteger contra o estresse ambiental e desidratação (VU *et al*., 2009). Devido à sua importância na formação do biofilme, a produção EPS tem sido alvo de diversas pesquisas para impossibilitar o processo de formação e de maturação dessas comunidades microbianas (MURRAY *et al*., 2009; NAGORSKA *et al*., 2010; VU *et al*., 2009).

II.1.7 Estratégias de Combate aos Biofilmes

Segundo Bjarnsholt e colaboradores (2013), quatro estratégias básicas podem ser utilizadas para se combater os biofilmes microbianos. São elas: prevenção, enfraquecimento, disrupção e morte direta (FIGURA 7). Entretanto, o autor enfatiza que, provavelmente, a combinação de mais de uma estratégia seja o meio mais efetivo de eliminação de biofilmes patogênicos.

A melhor ferramenta contra as infecções por biofilmes ainda é a prevenção, geralmente pela morte da bactéria ainda em seu estado planctônico e se apresenta muito mais susceptível à ação de antibióticos. Além disso, outro método preventivo é o impedimento da adesão inicial, permitindo que as defesas do hospedeiro possa atuar com sucesso e também possíveis antibióticos possam atuar com eficácia máxima. Entretanto, como, em geral, a

Figura 7: Métodos de combate ao biofilme microbiano.



Fonte: BJARNSHOLT *et al*., 2013.

formação inicial do biofilme no sítio de infecção gera mínima ou nenhuma inflamação, é praticamente impossível para o sistema imune detectar as bactérias iniciais. Quando o processo inflamatório é detectado, o biofilme já se encontra devidamente formado e não pode mais ser erradicado (BJARNSHOLT *et al*., 2013). Contudo, algumas técnicas já são utilizadas para prevenção de adesão bacteriana na superfície de cateteres e implantes como o recobrimento e condicionamento de superfície com substâncias antimicrobianas ou que ocupem os sítios de ligação de superfície (ESMAEILI; GHOBADIANPOUR, 2016; CHEN *et al*., 2016).

Nos casos em que a prevenção do biofilme não pôde ser alcançada, outra manobra é enfraquecer as bactérias que compões o biofilme através da neutralização de seus fatores de virulência e outras propriedades pró-formação de biofilmes como quorum sensign, sRNAs ou metabolismo de ferro. Contudo, estratégias que visem os fatores de virulência só se mostram efetivos nos estágios iniciais de infecção, ja que os mesmos são perdidos durante a adaptação bacteriana. Também é importante salientar que muitas dessas técnicas são espécie- ou cepa-específica (BJARNSHOLT *et al*., 2010).

Como discutido anteriormente, uma vez o biofilme estabelecido, as bactérias em seu interior são muito mais tolerantes à antibióticos do que bactérias planctônicas. A disrupção da comunidade pode reverter a tolerância física que a bactéria adquire em um biofilme. Como a matriz extracelular polimérica é responsável por estabilizar o biofilme, objetivar a produção dessa matriz ou seu rompimento pode enfraquecer a estrutura do biofilme, o que permitirá uma maior eficácia de antibióticos, aumento de fagocitose ou a liberação de bactérias do biofilme (BJARNSHOLT *et al*., 2013). Já é sabido que a maneira mais eficiente para a disrupção de um biofilme é através de métodos mecânicos ou cirúrgicos, mas tais métodos podem levar a uma disseminação da infecção. Dessa forma, outra forma de disrupção é a aplicação de enzimas que degradem a matriz do biofilme, como as DNAses inaláveis que são aprovadas e utilizadas para o tratamento de infecções crônicas dos pulmões como nos pacientes com fibrose cística (FREDERIKSEN *et al*., 2006).

Por fim, a morte direta do patógeno dentro do biofilme é a última estratégia a ser discutida. O uso de elevadas doses de antibióticos ou a combinação destes com as técnicas discutidas anteriormente são os métodos mais utilizados na atualidade. Contudo, o uso de altas doses de drogas pode acarretar no aumento de seus efeitos adversos e citotoxicidade, enquanto que a combinação de técnicas deve ser muito bem estudada pois a interação entre substâncias pode gerar dificuldades no seu manejo (BJARNSHOLT *et al*., 2013).

II.2 CÁRIE DENTAL

II.2.1 Conceito e Fatores Etiológicos da Cárie Dental

O termo Cárie Dental surgiu na literatura científica por volta de 1676 de acordo com o dicionário médico Merriam-Webster. É derivado do latim "caries" que significa decair, apodrecimento ou queda; e do antigo irlandês "ara-chrinn" que significa ele decai. O termo antigamente era utilizado para determinar os orifícios que apareciam nos dentes sem quaisquer conhecimento preciso de sua etiologia ou patogênese da doença em si.

Com o passar do tempo, o termo cárie foi empregado de forma genérica e ampla para descrever diversos tipos de lesões que tivessem em comum a formação de cavidades na estrutura dentária em quaisquer estado de desenvolvimento (BOWEN, 2015).

Appleton, em 1944, destacou que o uso inapropriado do termo cárie era comum, dessa forma, era necessário diferenciar-se o processo de formação da lesão, da cavidade formada em si. Portanto, o termo Cárie Dental deve ser utilizado estritamente para a doença em si, envolvendo seus aspectos etiológicos e patogênese, enquanto que as cavidades oriundas do processo deveriam ser tratadas como lesões cariosas (BOWEN, 2015; SIMÓN-SORO; MIRA, 2015).

Atualmente se é aceito e estabelecido universalmente que a cárie dentária é uma doença multifatorial, infecciosa, transmissível e dieta dependente, que produz uma desmineralização das estruturas dentárias de forma gradual (LIMA, 2007).

Segundo o mesmo autor, deve-se procurar um conhecimento mais aprofundado dos diversos fatores etiológicos envolvidos no processo de formação da cárie que possibilite estabelecer estratégias preventivas mais eficientes, sem que se corra o risco de promover algum desequilíbrio com consequências piores do que a própria "doença" (LIMA, 2007).

II.2.1.1 Microbioma

A primeira proposição acerca da relação do aspecto microbiológico como fator etiológico do processo cariogênico é antiga (BLACK, 1898; WILLIAM, 1897). Contudo, a confirmação de que bactérias orais, quando em ambiente com excesso de açúcares e outros fatores, são capazes de desenvolver a lesão de cárie só foi confirmada alguns anos depois por Miller (MILLER, 1902).

Durante o século XX, dentre todos os organismos associados a aparição da cárie dental, os estreptococos do grupo mutans despertaram grande interesse por parte dos pesquisadores ao redor do mundo (AL-DAJANI; LIMEBACK, 2012; BOWEN; KOO, 2011; PARISOTTO *et al*., 2011). Tal período de pesquisas chegou ao seu auge quando pesquisadores recolheram cepas de *Streptococcus mutans* isoladas inicialmente de lesões cariosas humanas e as utilizaram em modelos cariogênicos murinos, estabelecendo-se o papel crucial dessa espécie no processo de formação da cárie (FITZGERALD; KEYES, 1960; ORLAND *et al*., 1954). Contudo, deve-se ressaltar que a cárie é uma doença de cunho microbiano, mas que só ocorre quando da alteração em proporção da microbiota residente/patogênica associada a fatores ambientais (ZERO *et al*., 2009).

Apesar da participação inquestionável do *S. mutans* (SM) no microbioma da cárie, acredita-se atualmente que este microorganismo não seja o único responsável pelo processo. Diversos estudos clássicos já demonstraram que a quantidade de *S. mutans* não está necessariamente elevado em biofilmes associados à cáries, principalmente quando as lesões estão em estágios iniciais ainda sem cavitação (VAN HOUTE *et al*., 1991; SANSONE *et al*., 1993). Ao invés do *S. mutans*, foi proposto que bactérias acidogênicas e acidúricas "não-SM" e bactérias do gênero *Actinomyces* estariam muito mais envolvidas com a iniciação da cárie. Além disso, van Ruyven e colaboradores, em 2000, detectaram bactérias acidúricas "não-SM" como *Actinomyces, Lactobacillus* e *Bifidobacterium* em lesões cervicais de mancha branca de crianças e adolecentes.

Embora o *S. mutans* seja um dos microorganismos cariogênicos mais amplamente pesquisados, estudos ressaltam que ele é apenas um dentre mais de 1000 outras espécies encontradas no biofilme oral (WADE, 2013). Em estudos realizados através de identificação molecular bacteriana, pesquisadores relatam que comunidades bacterianas distintas, inclusive algumas novas espécies, estão associadas com o processo cariogênico e que *S. mutans* não é detectável em 10 - 20% dos indivíduos com cáries extensas (AAS *et al*., 2008; BEIER; QUIVEY; BERGER, 2012). Evidências recentes também elucidaram o papel de algumas leveduras, como a *Candida albicans*, como um membro da microbiota mista envolvida no aparecimento da cárie (KLINKE *et al*., 2009).

II.2.2. Epidemiologia

Durante as últimas décadas, houve um consenso mundial embasado em diversos relatos de que a cárie dental estava declinando de forma significativa em populações de todo o

globo (HAN; KIM; PARK, 2010; LAURIS; BASTOS; BASTOS, 2012). A comunidade odontológica se motivou através dos recursos utilizados para tal redução como o uso sistêmico e tópico de fluoretos, cremes dentais, selantes oclusais, melhora na qualidade da dieta, educação em saúde bucal e cuidados em prevenção (BAGRAMIAN; GARCIA-GODOY; VOLPE, 2009).

Contudo, alguns estudos reportam um aumento alarmante no número de pacientes acometidos por lesões cariosas em determinadas partes do mundo. Esse aumento acomete tanto crianças como adultos, dentição decídua e permanente, e superfícies coronárias e radiculares (HALCROW *et al*., 2013; ISAKSSON *et al*., 2013). As questões emergentes no que se refere a cárie dentária são comumente relacionadas com as disparidades existentes nos diversos programas de saúde pública ao redor do mundo, o que converge em diferentes quadros epidemiológicos da doença (FRAZÃO, 2012; GREWAL; VERMA; KUMAR, 2011).

Um dos principais fatores que merece destaque é o impacto social no panorama da cárie dental para grupos específicos ao redor do mundo. Os aumentos do número de lesões de cárie ocorrem em grupos de menor poder socioeconômico, imigrantes e crianças. Muito embora as causas para tal aumento não sejam precisas, é possível concluir que os benefícios da prevenção não estejam atingindo tais grupos (BAGRAMIAN; GARCIA-GODOY; VOLPE, 2009).

O último levantamento realizado pela Organização Mundial de Saúde (OMS) (2011) através da Universidade de Malmö, evidenciou um aumento significativo do índice de Dentes Perdidos, Cariados ou Obturados (CPO-D) de 1,61, em 2004, para 1,67 (NATARAJAN, 2011). Apesar de aparentemente pequeno, esse aumento indica que possíveis problemas, no que se refere ao âmbito da prevenção, possam estar acontecendo (FRAZÃO, 2012).

Quando o mesmo levantamento é separado de acordo com os continentes avaliados, percebe-se que apenas os continentes da Europa e América do Norte apresentaram reduções nos seus índices CPO-D, enquanto que, nos outros continentes, é possível evidenciar que os índices de CPO-D aumentaram (QUADRO 1) (NATARAJAN, 2011).

Referente ao Brasil, o mesmo se enquadra, segundo classificação proposta pela OMS, com índices de prevalência moderados (TABELA 1) (BRASIL, 2011). Segundo alguns autores, essa melhora na qualidade da saúde bucal dos brasileiros se deve a implementação de diversas medidas de saúde pública no âmbito da prevenção (ALMEIDA *et al*., 2012; TRAEBERT *et al*., 2009).

Quadro 1: Índices de CPO-D em indivíduos de 12 anos especificados por região do globo.

CONTINENTES ANALISADOS	ÍNDICE CPO-D	
	2004	2011
África	1,15	1,19
América do Norte	2,76	2,35
América do Sul	1,58	1,63
Europa	2,57	1,95
Ásia	1,12	1,87
Oceania	1,39	1,48

Fonte: NATARAJAN, 2011.

Tabela 1: Média do Índice CPO-D para o grupo etário de 12 anos e proporção dos componentes em relação ao CPO-D total, segundo regiões.

			Hígido	Cariado		Obt/Cariado		Obturado		Perdido		CPO-D
	Região	N	Média	Média	%	Média	%	Média	%	Média	%	Média
12 anos de idade	Norte	1.703	22,69	2,13	67,4	0,14	4,4	0,65	20,6	0,24	7,6	3,16
	Nordeste	2.021	23,33	1,81	68,8	0,10	3,8	0,50	19,0	0,22	8,4	2,63
	Sudeste	1.339	23,30	0,78	45,3	0,07	4,1	0,77	44,8	0,11	6,4	1,72
	Sul	1.005	23,00	1,13	54,9	0,12	5,8	0,76	36,9	0,04	1,9	2,06
	Centro-Oeste	1.179	22,75	1,52	57,8	0,15	5,7	0,87	33,1	0,09	3,4	2,63
	Total	**7.247**	**23,18**	**1,12**	**54,1**	**0,09**	**4,3**	**0,73**	**35,3**	**0,12**	**5,8**	**2,07**

Fonte: BRASIL, 2011.

Todavia, dentro do próprio país, tem-se que regiões se apresentam com índices moderados e outros com índices altos, segundo a OMS (TABELA 1) (BRASIL, 2011). Esse panorama retrata a própria desigualdade de acesso à atenção básica e prevenção entre as regiões do país, dentre elas, destacam-se as regiões Norte, Nordeste e Centro-Oeste com os piores índices do país. Muito embora os índices referentes a essas regiões tenham declinado quando comparados com os de 2007, muito ainda precisa ser feito para que o Brasil alcance título de "país com baixo índice de cárie" (BRASIL, 2011).

II.2.3 Processo Cariogênico

O processo de desenvolvimento das lesões cariosas é definido como dinâmico, complexo e, como já abordado, multifatorial. Para melhor se compreender o aparecimento de

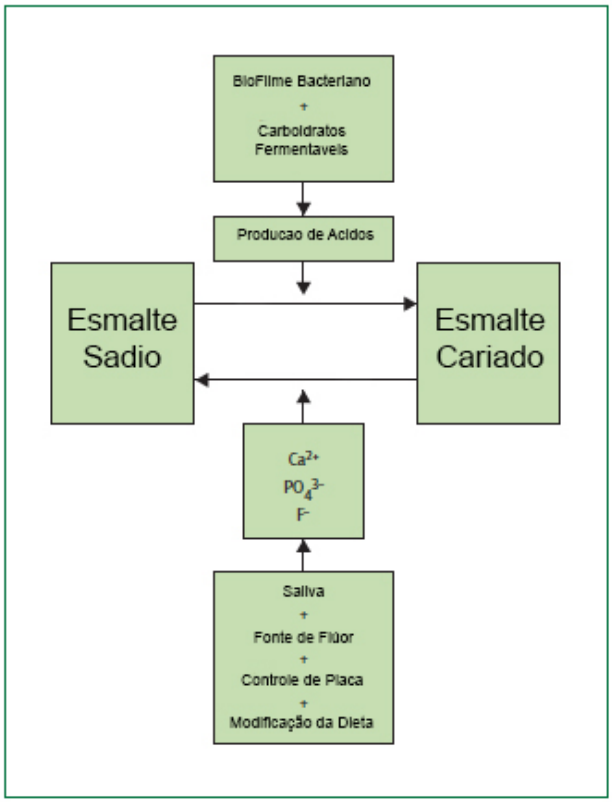
cavitações em estrutura dentária, deve-se ter em mente que o elemento dentário sofre constantes processos de desmineralização e remineralização, comumente denominado de processo des-re (CURY; TENUTA, 2009).

A cavidade oral, por ser um ambiente extremamente dinâmico, sofre constantemente variações de pH, sejam estas decorrentes do processo de alimentação ou refluxo do suco gástrico. As variações que induzem pHs ácidos acarretam alterações químicas e bioquímicas nas nanoestruturas que compõem tanto o esmalte quanto a dentina. Dessa forma, quando sítios susceptíveis são submetidos ao ataque de ácidos, os íons cálcio e fosfato responsáveis pela mineralização dos cristais de hidroxiapatita são solubilizados no meio bucal (FIGURA 9) (FEATHERSTONE, 2008).

Contudo, tal processo ocorre rotineiramente e de forma rápida no meio oral, pois, devido à capacidade tampão da saliva, o pH rapidamente é restaurado ao seu índice fisiológico e a etapa de reparo (remineralização) se inicia. Nessa etapa, ocorre o caminho inverso, no qual os íons cálcio e fosfato solubilizados no meio oral são depositados na estrutura dentária (FEATHERSTONE, 2008) (FIGURA 9).

No processo cariogênico, ocorre um desequilíbrio dessa equação formada entre as variáveis desmineralização e remineralização, em que, devido ao acúmulo bacteriano na superfície do dente e a liberação de produtos ácidos decorrentes da fermentação de açúcares, o processo de desmineralização é acentuado e acontece de forma contínua (CURY; TENUTA, 2009). Nessa etapa, além da solubilização da matriz composta por diversos minerais, ocorre a solubilização dos cristais de hidroxiapatita, principal componente do esmalte dentário.

Figura 9: Diagrama esquemático do processo de desmineralização e remineralização.

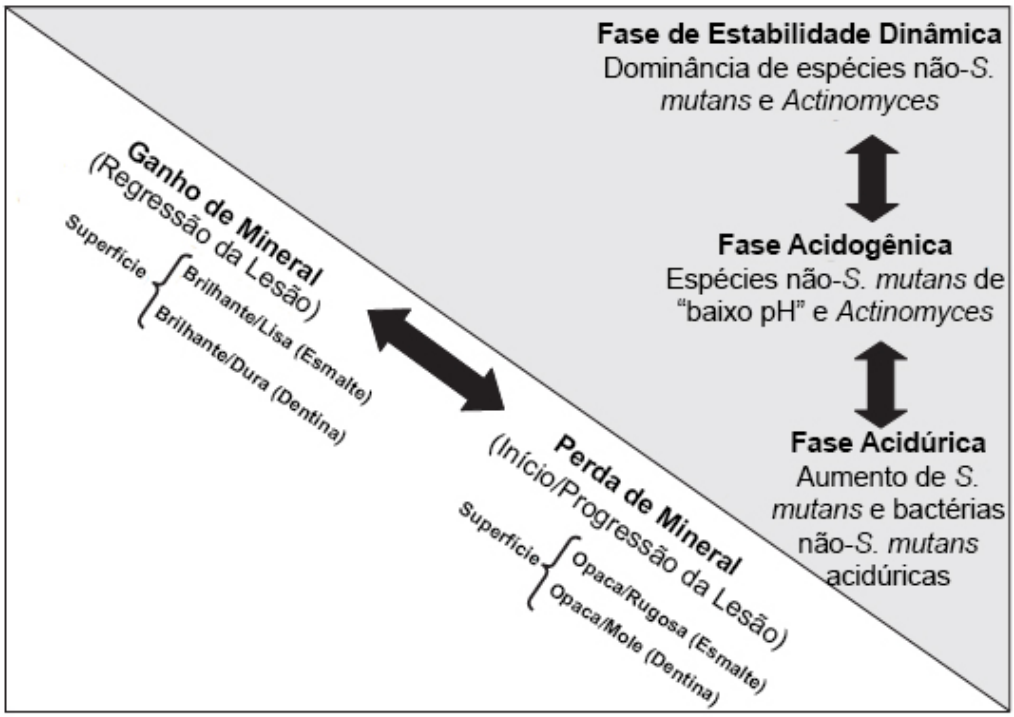


Fonte: SELWITZ; ISMAIL; PITTS, 2007 com modificações.

Estudos demontram que os colonizadores iniciais das superfícies dentárias recentemente higienizadas

constituem uma parte altamente selecionada do microbioma oral, principalmente *S. sanguinis, S. oralis* e *S. parasanguinis,* mas outros gêneros como Actinomyces também estão presentes (LI *et al*., 2004; DIGE *et al*., 2009). Surpreendentemente, *S. mutans* é responsável por apenas 2% ou menos da população inicial de streptococcus, independentemente da atividade de cárie do indivíduo (NYVAD; KILIAN, 1990). Tais achados enfatizam o fato que a grande maioria dos colonizadores iniciais são do grupo mitis. Essa fase é denominada de "Fase de Estabilidade Dinâmica" (FIGURA 10) (TAKAHASHI; NYVAD, 2011).

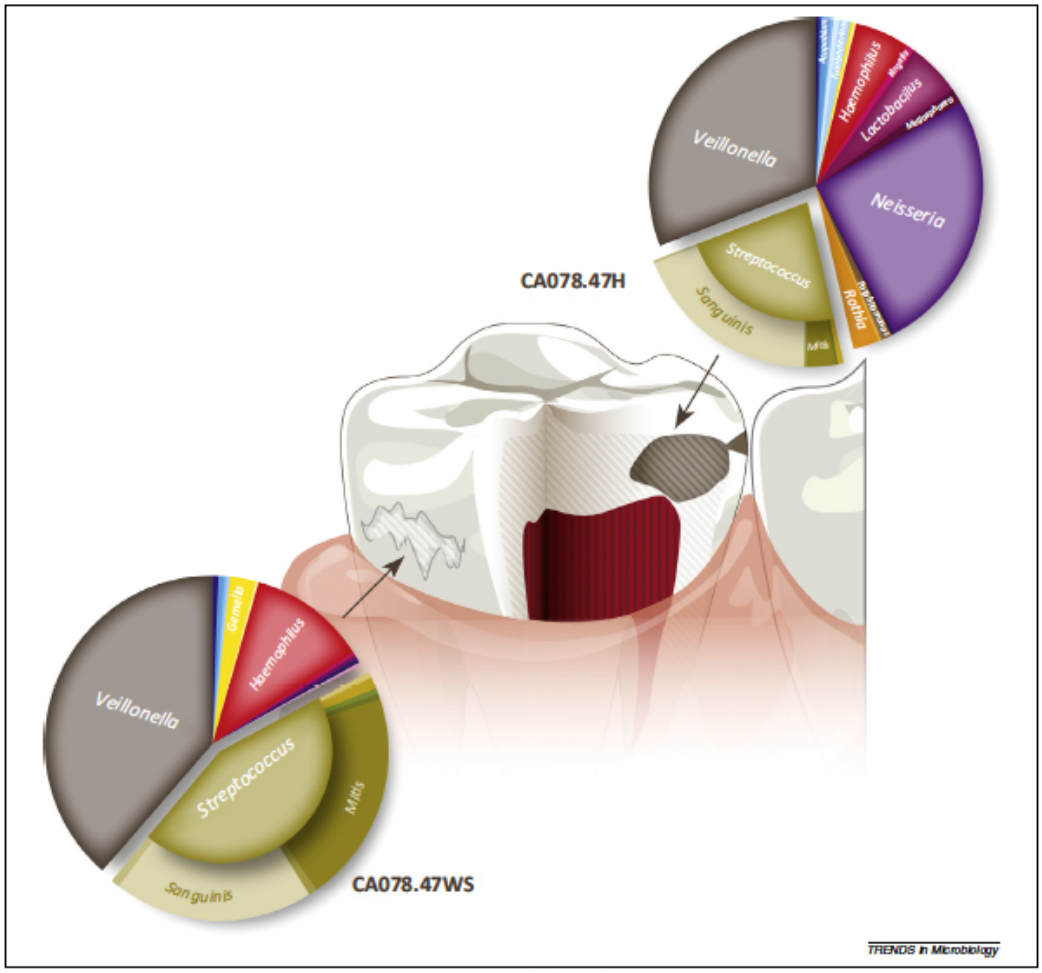
Figura 10: Diagrama esquemático das fases de desenvolvimento ecológico do biofilme cariogênico e das aparências clínicas associadas à progressão da lesão.



Fonte: TAKAHASHI; NYVAD (2011) com modificações.

A partir do envelhecimento do biofilme, a composição bacteriana muda para uma dominância de *Streptococcus* e *Actinomyces*. Contudo, a proporção de Streptococcus é extremamente superior, apesar da quantidade de *S. mutans* ainda ser consideravelmente baixa. Tais *Streptococcus* não-mutans, quando associados com Lactobacilos, exacerbam o poder acidogênico do biofilme, o que gera uma queda inicial do pH no local e a formação de lesões de mancha branca em esmalte (FIGURA 11). Esta fase é chamada de "Fase Acidogênica" (FIGURA 10) (TAKAHASHI; NYVAD, 2011).

Figura 11: Desenho evidenciando a diferença na microbiota em diferentes estágios da lesão cariosa.



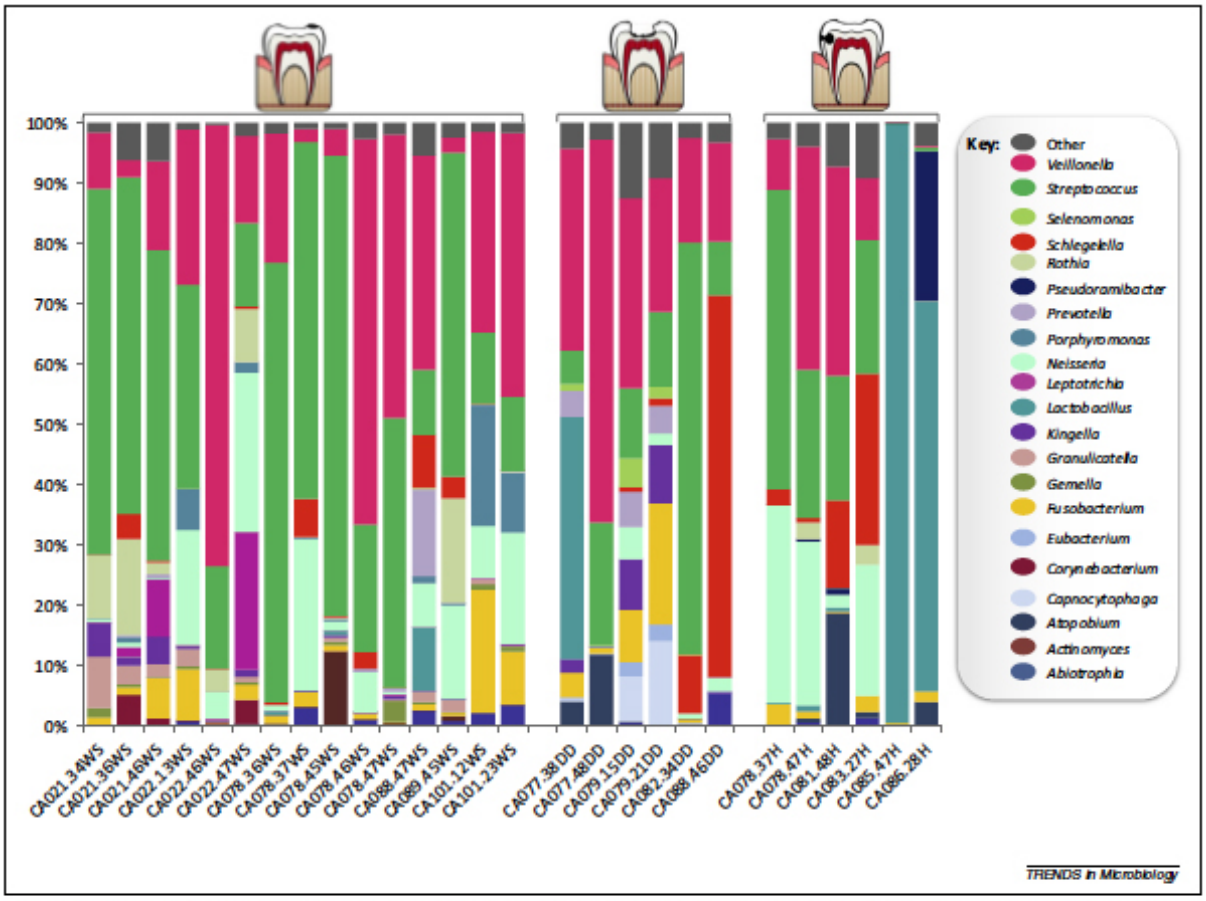
Fonte: SIMÓN-SORO; MIRA (2015).

A última fase do processo, Fase Acidúrica, é marcada pela mudança quase que radical na composição bacteriana do biofilme (FIGURA 10). Nessa fase, já existe lesão cariosa em dentina e cerca de 30% da microbiota total do biofilme é composta por *S. mutans*, sugerindo que essa espécie está diretamente asociada com os estágios progressivos da cárie (FIGURA 11). Entretanto, a medida que a lesão cariosa aumenta e o pH cai mais drasticamente, cria-se o ambiente perfeito para o desenvolvimento de espécies até então quase inexistentes no local como *Prevotellae* e *Bifidobacterium*. Essas bactérias possuem um potencial acidogênico muito superior ao *S. mutans* e geram um microambiente inóspito ao *S. mutans,* fazendo que a quantidade de células dessa espécie diminua a medida que a lesão aumenta (TAKAHASHI; NYVAD, 2011).

Todo esse processo de sucessão ecológica bacteriana mostra claramente como a microbiota na superfície dental muda ao longo da evolução da lesão. Na figura 12 pode ser visualizada a mudança de espécies e grupos bacterianos ao longo de diversas fases da lesão

cariosa. Tal estudo foi realizado por Simón-Soro e colaboradores em 2014 e a imagem reproduzida pelos mesmos autores em 2015.

Figura 12: Análise por metatranscriptômica dos grupos bacterianos presentes em biofilmes associados a diferentes estágios de cárie (lesão de mancha branca, lesão cariosa em dentina e lesões mais profundas com acometimento pulpar).



Fonte: SIMÓN-SORO; MIRA (2015).

II.2.4 Prevenção

É sabido que a doença cárie é causada por uma inter-relação de diversos fatores que culminam na desmineralização dental (LIMA, 2007). Por ser considerada uma lesão de caráter crônico e irreversível, grande foco tem sido dado nos últimos anos para sua prevenção, haja visto que as técnicas intervencionistas convencionais não são capazes de devolver ao elemento acometido suas propriedades originais (PALOMBO, 2011).

Dessa forma, em virtude do caráter microbiológico associado à lesão, diversos autores enfatizam a importância da escovação e remoção mecânica dos biofilmes associados ao elemento dentário como modo mais eficaz para diminuição dos índices de cárie (CHOI *et al*., 2012; LIU *et al*., 2010). Entretanto, tal afirmativa ainda é motivo de discussão ao redor do

mundo, devido ao fator "cooperação" intimamente atrelado ao indivíduo. Esse fator faz alusão aos déficits existentes na rotina de higiene oral dependentes de aspectos culturais, sociais e econômicos (LIU *et al*., 2010; PENGPID; PELTZER, 2012).

Portanto, com o passar dos anos, outros métodos de controle de cárie foram desenvolvidos, de forma que os mesmos dependessem cada vez menos da cooperação do paciente, ou, ao menos, fossem mais eficazes quando das variações de utilização. Dentre esses, pode-se destacar o gluconato de clorexidina.

II.2.4.1 Gluconato de clorexidina

O gluconato de clorexidina é um agente antimicrobiano com ampla atividade sobre diversos microorganismos, dentre os quais várias bactérias patogênicas de cavidade oral, e tem sido considerado como um potencial agente na prevenção da cárie dental (AUTIO-GOLD, 2008).

É considerado como agente padrão ouro contra diversas cepas microbianas orais ou de outros sítios tanto por possuir marcante atividade antimicrobiana, como também um efeito extremamente rápido, sendo capaz de gerar a morte bacteriana em apenas alguns minutos através tanto do comprometimento da membrana celular, como principalmente por interferência em vias metabólicas bacterianas essenciais (DOUGHTY-SHENTON *et al*., 2010; RODRIGUES *et al*., 2011). Apesar de possuir diversas formulações e vias de administração, o seu uso em Odontologia é restrito atualmente aos colutórios orais na concentração de 0,12%, vindo a ser inicialmente utilizado de forma comercial na década de 1980, nos Estados Unidos (MIKKELSEN *et al*., 1981).

Apesar de todas as suas vantagens, a clorexidina possui diversos efeitos adversos que limitam seu uso a médio/longo prazo. Dentre eles destacam-se a alteração de cor dos dentes, perda do paladar, ardência bucal, xerostomia e descamação da mucosa oral (AUTIO-GOLD, 2008). Dessa forma, o uso da clorexidina na cavidade oral tem sido direcionado para casos específicos em que o paciente apresenta quadros infecciosos mais graves ou apresenta dificuldades na higienização oral (VAN STRYDONCK *et al*., 2012).

Nos últimos anos estudo apontam a importância da descoberta de outras moléculas tão ativas quanto a clorexidina, haja visto que determinadas cepas bacterianas já apresentam elevada resistência a essa substância (PALOMBO, 2011). Além disso, o elevado número de efeitos adversos associados ao uso contínuo da clorexidina inviabilizam esse agente como forma de prevenção da cárie dentária (JAMES; PARNELL; WHELTON, 2010).

II.3 LESÕES ENDODÔNTICAS

II.3.1 Aspectos Gerais

Durante os últimos anos, avanços significativos foram realizados no estudo da cárie dental, sua progressão e seus efeitos sobre os tecidos dentais. Como abordado anteriormente, a cárie dental inicia-se como lesões de mancha branca, as quais são caracterizadas por um esmalte rugoso e opaco de forma localizada. A medida que a doença evolui, o tecido dentinário pode ser acometido e, em última instância, a polpa dentária é afetada (FABREGAS; RUBINSTEIN, 2013).

Quando o tecido pulpar é acometido seja de forma direta, através da comunicação da lesão de cárie com a câmara pulpar; ou indireta, pela penetração bacteriana nos túbulos dentinarios alcançando o tecido mole, o mesmo inicia um processo inflamatório localizado. A inflamação do tecido pulpar é denominada pulpite (TASCHIERI *et al*., 2014).

A pulpite, a depender do caso clínico, pode ser considerada reversível, quando ainda é possível a remoção dos fatores agressores e o retorno da saúde do tecido; ou irreversível, nos casos em que o tecido pulpar está em um quadro inflamatório mais avançado e/ou o quadro infecciosos já se alastrou. Neste último quadro, mais comumente, o tecido pulpar evolui para a necrose, fomentando a disseminação microbiana pelos canais radiculares em direção apical (PARK *et al.,* 2015).

A endodontia é a especialidade odontológica responsável por estudar e desempenhar o controle e prevenção das infecções pulpares e perirradiculares (VIEIRA *et al*., 2011). Dessa forma, nos casos citados acima, o tratamento endodôntico geralmente é realizado com o objetivo de remover o tecido pulpar contaminado e descontaminar toda a extensão radicular e periapical dentária (PLOTINO *et al.,* 2016).

Muito embora outros fatores possam levar à necessidade de tratamento endodôntico, como os traumatismos dento-alveolares, é consenso científico de que a infecção microbiana oriunda das lesões cariosas é o prelúdio para o desenvolvimento de pulpites e lesões endodônticas (ZEHNDER; BELIBASAKIS, 2015; ANDREASEN; KAHLER, 2015; TASCHIERI *et al*., 2014).

II.3.2 Epidemiologia

Até a presente data não existem dados consolidados acerca das taxas de prevalência e incidência de lesões endodônticas no mundo. Além disso, os poucos dados existentes ainda se apresentam com uma alta heterogeneidade ao redor do globo.

Estrela e Bueno (2008) em seu levantamento bibliográfico relatam que estudos epidemiológicos associados com a endodôntia são menos comuns que em outras especialidades, como a periodontia e a cariologia, e que estes foram inicialmente observados em investigações escandinavas.

Os mesmos autores destacaram ainda a variabilidade nas taxas de prevalência de tratamentos endodônticos dentre as populações e estudos avaliados (entre 1,2 - 26%). Tal variação pode ser justificada pelas diferentes metodologias utilizadas nas respectivas pesquisas. Diferenças populacionais, no método de diagnóstico endodôntico ou até mesmo na precisão do avaliador podem ser responsáveis por esse amplo intervalo (ESTRELA; BUENO, 2008).

No Brasil, o levantamento nacional mais recente acerca da necessidade de tratamento endodôntico em diversas faixas etárias da população foi publicado em 2010 pelo Ministério da Saúde. No relatório final do projeto SB Brasil, foi verificado que, em média, 0,4% da população apresentava necessidade de tratamento endodôntico. Apesar do índice nacional relativamente baixo, as regiões Norte e Nordeste do país apresentaram as maiores médias (1% e 0,5% respectivamente) (BRASIL, 2011). Tal fato se deve, provavelmente, ao menor nível de acesso à estratégias de prevenção à carie e ao atendimento de saúde. Além disso, outro viés possível para os baixos valores nacionais encontrados se deve ao alto número de exodontias e a cultura ainda extracionista da população e profissionais brasileiros (SOUSA et al., 2013).

II.3.3 Patogênese das Lesões Endodônticas

II.3.3.1 Microbioma

A infecção endodôntica primária é considerado um processo dinâmico, no qual as espécies bacterianas dominantes variam através dos diferentes estágios da doença. Através de estudos experimentais, foi possível estabelecer os principais fatores que norteiam o processo de colonização endodôntica e causam a intensa variação na microbiota. São eles: a

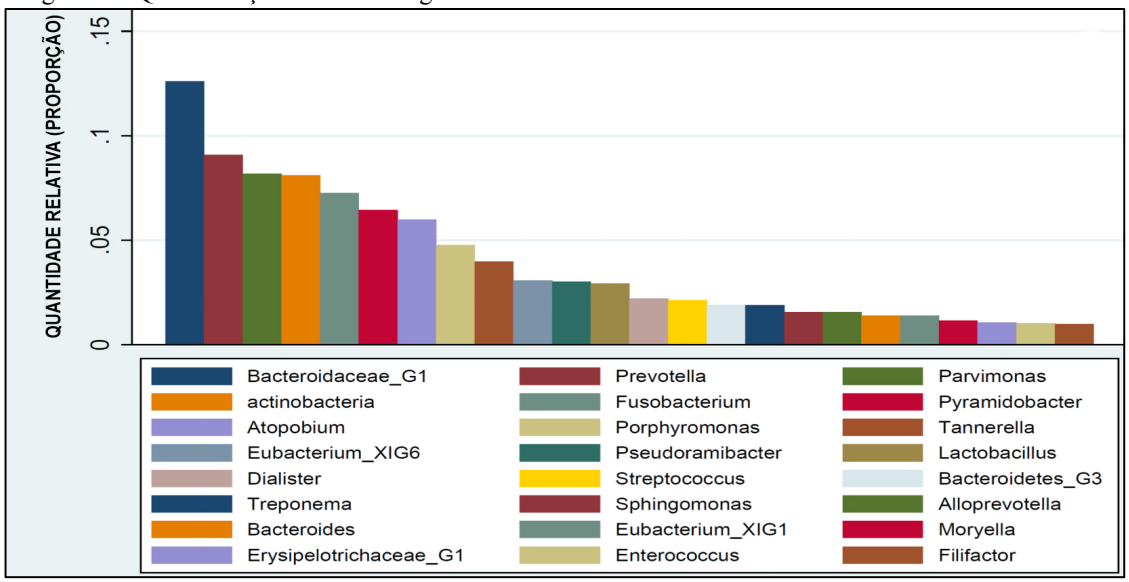
disponibilidade de nutrição; níveis de oxigênio; e o pH local nas diferentes porções radiculares (SANTOS et al., 2011).

Nos casos de tratamentos endodônticos primários, nos quais existe uma comunicação direta entre o conjunto de canais radiculares e o meio bucal, há uma predominância de microorganismos anaeróbios facultativos e aeróbios. Tais dentes são considerados mais "resistentes" ao tratamento em virtude da alta concentração de bactérias entéricas no canal radicular (SIREN et al., 1997).

No mesmo estudo foi realizada a coleta de microorganismos oriundos do canal radicular de diferentes pacientes em diferentes momentos do tratamento endodôntico. Foi percebido que em 55% dos dentes que estavam abertos primariamente ao tratamento apresentavam bactérias do grupo dos *Enterococci*. Além disso, notou-se também que, mesmo após a 10ª visita para troca de medicação, ainda se encontravam quantidades relevantes de *Enterococcus faecalis* nos canais radiculares (SIREN et al., 1997).

Apesar desse resultado impactante, estudos mais recentes como o de Tzanetakis e colaboradores (2015) demonstram que, apesar do papel fundamental do *E. faecalis* nas infecções endodônticas primárias, ele pode não estar tão presente quanto se pensava. Neste estudo, através de análises moleculares, verificou-se a concentração bacteriana em 48 amostras bacterianas removidas de canais radiculares diferentes. Notou-se que, em geral, os filos Bacteroidetes, Firmicutes e Proteobacteria são os mais prevalentes em infecções primárias, enquanto que o gênero *Enterococcus* representou menos que 5% do total de espécies encontradas (FIGURA 15).

Figura 15: Quantificação relativa dos gêneros mais abundantes encontrados nas amostras coletadas.



Fonte: TZANETAKIS *et al.,* 2015 com modificações.

Contudo, quando abordam-se as infecções tardias ou secundárias (infecções persistentes mesmo após o tratamento concluído), o cenário é consideravelmente alterado. Embora o filo Proteobacteria esteja presente nas infecções primárias, ele é consideravelmente mais abundante nos casos de infecções persistentes. Além deste, os filos Tenericutes e Fusobacteria também estão significativamente em maior concentração (TZANETAKIS *et al.,* 2015). Tais achados demonstram que, ao contrário do que se pensava em décadas passadas, as infecções persistentes são polimicrobianas e, geralmente, derivam de tratamentos primários inadequados ou agentes químicos ineficientes (TZANETAKIS *et al.,* 2015; ZAKARIA *et al*., 2014; SAKAMOTO *et al*., 2008).

Ainda nos casos de infecções persistentes, uma das espécies mais estudadas é a *Enterococcus faecalis.* Apesar do fato que em determinados estudos a quantidade comparativa de *E. faecalis* seja inferior à outros microorganismos (TZANETAKIS *et al.,* 2015), esta espécie é encontrada em 23-70% dos canais radiculares obturados com espessamento periapical (WANG *et al.,* 2012; ANDERSON *et al.,* 2012; HANCOCK *et al.,* 2001).

Estudos que investigam a ocorrência da *E. faecalis* nos canais radiculares de dentes já tratados e com espeçamento periapical confirmam que as habilidades dessa bactéria de resistir aos antimicrobianos convencionais e de se adaptar às diversas mudanças ambientais são fundamentais para sua sobrevivência nos canais radiculares e causa das re-infecções (MALLICK *et al.,* 2014; KHALIFA *et al.,* 2015).

Enterococcus faecalis é uma bactéria que possui um arsenal gênico bem diversificado e propício para se desenvolver em ambientes inóspitos. Esta espécie possui mecanismos de adesão como moléculas de agregação, carboidratos de superfície ou proteínas de adesão à fibronectina que permitem uma melhor união da bactéria às moléculas de colágeno Tipo I e proteínas de matriz presentes na dentina radicular (PENG *et al.,* 2014; JIANG; YAN; LIANG, 2014).

Além disso, a produção de toxinas como a citolisina podem induzir a destruição tecidual (OGAKI *et al.,* 2016), enquanto bacteriocinas como a AS-48 é capaz de inibir o crescimento de outros microorganismos (CEBRIÁN *et al.,* 2015). Não obstante ao mecanismos até agora apresentados, o *E. faecalis* ainda é capaz de produzir radicais superóxido que é capaz de modular o processo inflamatório pulpar e estimular a liberação de prostaglandinas, interleucinas e fator de necrose tumoral que contribuem para o dano tecidual periradicular (GRAVES; OATES; GARLET, 2011).

Dentro do repertório de fatores de virulência do *E. faecalis* tem-se ainda a secreção da enzima hialuronidase, responsável por facilitar a disseminação bacteriana bem

como suas toxinas, e proteases como a gelatinase que contribui para a reabsorção óssea e degradação da matriz orgânica da dentina (BISWAS *et al.,* 2016).

Por fim, o *Enterococcus faecalis* possui uma elevada facilidade para coagregação e formação de biofilmes na superfície dentinária. Tal característica é responsável por permitir uma resistência de até 1000 vezes à fagocitose, anticorpos e agentes antimicrobianos, quando comparada com outras células em estado planctônico (MALLICK *et al.,* 2014).

II.3.3.2 Resposta imunoinflamatória

O processo inflamatório endodôntico relacionado à infecção envolve primeiramente a polpa dental e, somente depois, se instala na região periapical. O tecido pulpar pode, geralmente, ser considerado um tecido imunocompetente com capacidade de resposta à estímulos nocivos (RICUCCI *et al.,* 2009).

Células dendríticas estão presentes na camada odontoblástica, além de fagócitos mononucleares serem encontrados na porção central do tecido pulpar. Uma pequena quantidade de células T recirculantes pode ser identificada, enquanto que células B são extremamente raras ou não presentes (CHAE *et al.,* 2002).

A medida que o processo de agressão tecidual se inicia através da liberação de toxinas bacterianas através dos túbulos dentinários, aumenta-se o influxoe de células polimorfonucleares (PMNs) e monócitos. A medida que a infecção progride, o infiltrado inflamatório se torna mais intenso e assume um perfil misto com a presença de células $TCD4^{+}$ e $TCD8^{+}$, células B e plasmócitos, juntamente com PMNs, monócitos, linfócitos Natural Killer e altos níveis teciduais de IgG e IgA (GRAVES; OATES; GARLET, 2011).

O processo inflamatório se expande com a atuação das aminas vasoativas (histamina e serotonina) que causam a dilatação e aumento da permeabilidade vascular culminando no edema pulpar e dor local. A secreção e ativação do sistema calicreína-cinina também é exacerbado nesse momento contribuindo para os eventos recém descritos acima (GRAVES; OATES; GARLET, 2011).

Com o início do ciclo do ácido aracdônico a reação inflamatória chega no seu ápice. A produção de prostaglandinas, principalmente PGE2 e PGI2, tem sido correlacionada com o auge da inflamação, dor via mediadores químicos e posterior destruição tecidual pulpar (MCNICHOLAS *et al.,* 1991).

Caso o processo infeccioso não seja combatido e eliminado nessa etapa, o processo inflamatório progride para a necrose tecidual pulpar, disseminação bacteriana pelos canalículos radiculares e acometimento do tecido periradicular.

As lesões periapicais representam uma resposta imune localizada à infecção pulpar com a intenção de confinar os agentes agressores na região apical radicular. Em diversos aspectos o processo inflamatório apical é semelhante à inflamação pulpar descrita anteriormente, com a exceção de que, nesse momento, existe a destruição óssea associada (GRAVES; OATES; GARLET, 2011).

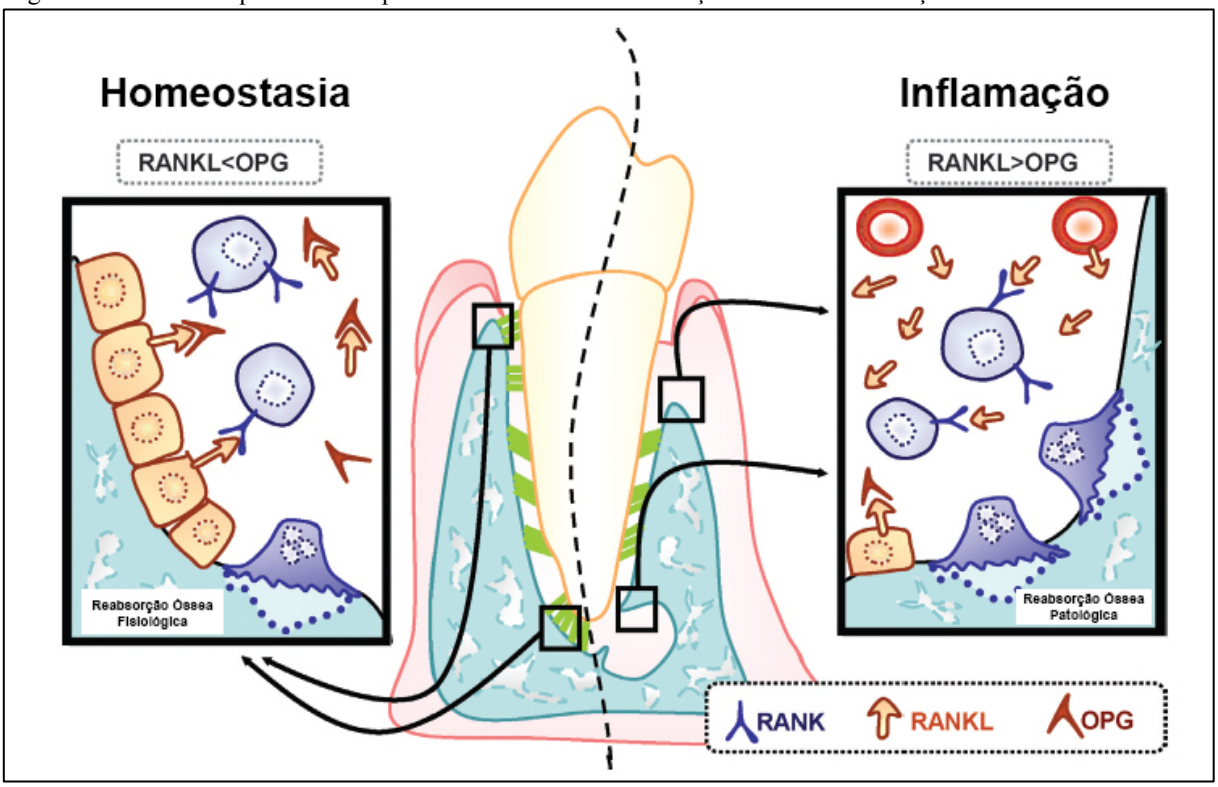
A inflamação periapical, em virtude das diversas prostaglandinas, principalmente PGE2, secretadas localmente, estimula a proliferação e ativação de osteoclastos que geram a destruição óssea periradicular. Muito embora esse processo só se inicie com necrose do tecido pulpar, é comum feixes nervosos ainda estarem vitais nessa região, culminando em um sinal clínico bastante comum que é a dor mesmo quando radiograficamente se é perceptível a visualização de lesões ósseas (MCNICHOLAS *et al.,* 1991).

Além da ativação de prostaglandinas, lesões endodônticas têm sido cada vez mais associadas com múltiplas citocinas e quimiocinas inflamatórias. Citocinas e suas rotas bioquímicas para promoção da reabsorção óssea podem ser visualizadas nas figuras 17 e 18.

O equilíbrio entre RANKL/OPG é um fator importante na regulação da reabsorção óssea nas regiões periapicais e periodontais. Diferenciação e ativação de osteoclastos são direcionados pela interação de RANK (Receptor Ativador do Fator Nuclear-kB) com seu ligante, RANKL. Osteoprotegerina, OPG, é um receptor competitivo para RANKL que inibe a ligação RANK-RANKL. Em situações de homeostase, RANKL e OPG estão equilibrados. Dessa forma existem taxas semelhantes de reabsorção e neoformação ósseas. Contudo, com o estímulo inflamatório, a quantidade de RANKL aumenta desequilibrando a balança para o lado da reabsorção óssea por osteoclastos (MENEZES *et al.,* 2008; COLIC *et al.,* 2009) (FIGURA 16).

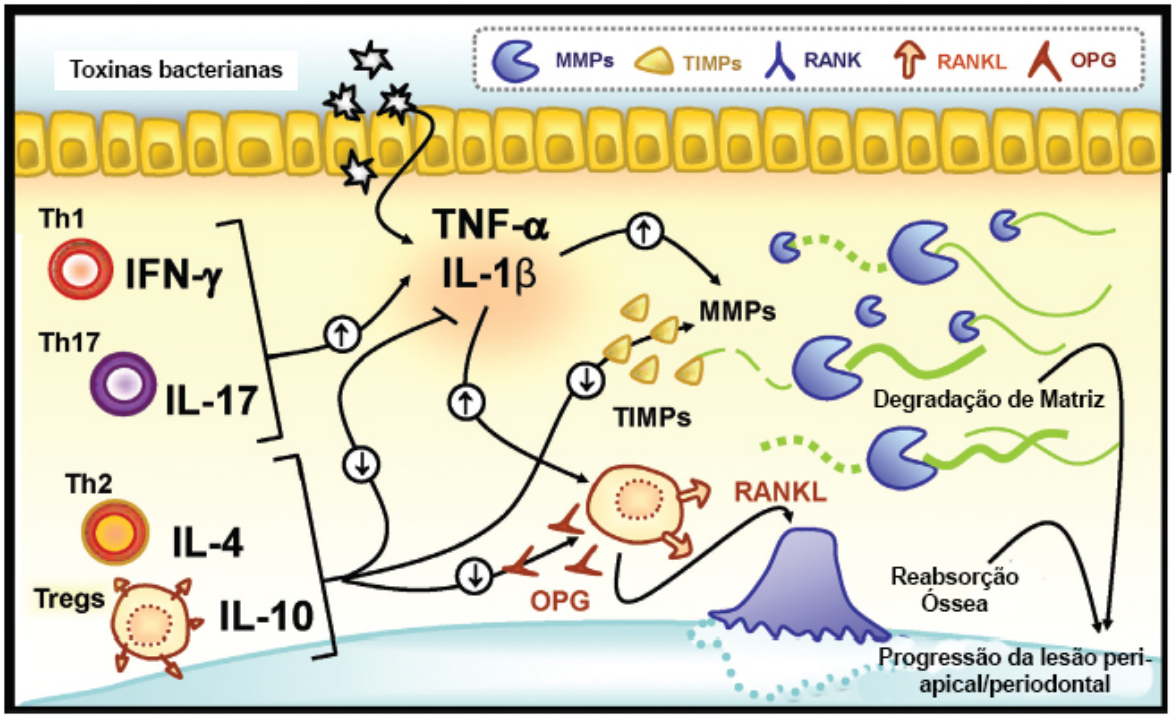
Dentre as citocinas que podem deflagrar a reabsorção óssea, IL-1 e TNF-α são os mais estudados na atualidade. Essas citocinas são capazes de estimular a degradação da matriz extracelular do tecido conjuntivo através da ativação de metaloproteinases de matriz (MMPs) (WALKER *et al.,* 2000). Além disso, TNF-α pode estimular a osteoclastogênese de forma direta ou associada com outras citocinas pró-inflamatórias como IL-6. A participação de células do sistema imune inato e adaptativo são fundamentais para esse processo (HUANG *et al.,* 2001) (FIGURA 17).

Figura 16: Modelo esquemático do processo reabsortivo em situações de saúde e doença.



Fonte: GRAVES; OATES; GARLET, 2011 com modificações.

Figura 17: Regulação de degradação de matriz tecidual e reabsorção óssea mediada por citocinas.



Fonte: GRAVES; OATES; GARLET, 2011 com modificações.

II.3.4 Modalidades de Tratamento Endodôntico

O tratamento endodôntico possui a finalidade principal de descontaminar a rede de condutos radiculares e permitir um reparo ósseo e tecidual adequado ao redor do dente acometido. Apesar de diversas possibilidades para se atingir esse objetivo, praticamente todas derivam da terapia clássica composta por uma fase de descontaminação mecânica e outra de descontaminação química (DARCEY *et al.,* 2015).

A preparação mecânica do dente e dos condutos radiculares possui dois objetivos principais. São eles:

- Facilitar a ação química: radiografias convencionais não permitem ao operador visualizar verdadeiramente a complexidade do sistema de canais radiculares. Canais laterais, acessórios, anastomoses e ramificações estão presentes em praticamente todas as situações (DARCEY *et al.,* 2015). Estudo comprova que, nos casos de deficiência de instrumentação, cerca de 53% dos condutos permanecem contaminados e não são completamente preenchidos com material obturador. Dessa forma, o preparo mecânico dos canais facilita a penetração de soluções irrigadoras , além de remover e desestruturar boa parte do biofilme aderido nas paredes dentinarias radiculares (PETERS *et al.,* 2001).
- Facilitar a obturação dos canais radiculares: como o debridamento mecânico e até mesmo o químico não são capazes de remover todos os microorganismos da região intracanal, a obturação visa o selamento de todos os canais visando o aprisionamento bacteriano e a diminuição de condições favoráveis ao seu desenvolvimento (DARCEY *et al.,* 2015).

Atualmente existem diversas técnicas de instrumentação e preparo que podem ser utilizadas a depender do caso clínico em questão. Desde o uso de instrumentos manuais flexíveis com diversos diâmetros e conicidades, até a utilização de instrumentos rotatórios que permitem uma maior agilidade no processo de preparo dos canais. Além de protocolos clínicos de descontaminação que podem seguir em direção coroa-ápice ou ápice-coroa com suas respectivas variações clínicas.

Contudo, como previamente descrito, a instrumentação mecânica necessita, obrigatoriamente, de uma terapia associada que vise a máxima descontaminação do sistema de canais radiculares. Em geral, tal terapia é a irrigação com produtos químicos

antimicrobianos, muito embora novas tecnologias venham sendo desenvolvidas em prol de maior índice de sucesso nos tratamentos (ZEHNDER, 2006).

O uso de irrigantes na Endodontia é uma prática corriqueira. Contudo, diversas soluções podem ser utilizadas de acordo com o caso, suas propriedades químicas, antimicrobianas e compatibilidade tecidual. Dentre as diversas finalidades da irrigação pode-se citar: a dissolução de toxinas bacterianas, remoção de restos necróticos pulpares, retirada de componentes inorgânicos oriundos do debridamento mecânico, bem como a morte direta das bactérias colonizadoras dos canais radiculares (FEDOROWICZ *et al.,* 2012).

Diversas soluções irrigadoras ou a combinação destas são utilizadas na prática da Endodontia. Dentre elas:

- Hipoclorito de Sódio;
- Clorexidina;
- Ácido Etilenodiamino Tetra-Acético (EDTA);
- Sterilox;
- Soluções iodadas;
- Peróxido de Hidrogênio;
- Anestésicos locais / água / solução salina fisiológica.

Dentre estas, o hipoclorito de sódio e a clorexidina são as soluções irrigadoras mais comumente utilizadas na prática clínica (VENKATESHBABU *et al.,* 2016).

O hipoclorito de sódio (NaOCl) foi primeiramente descrito como solução irrigadora em 1919 (COOLIDGE, 1919). Tal solução possui diversos atributos de um agente antimicrobiano ideal como sua ação rápida, amplo espectro de ação e é consideravelmente de baixo custo. Sua atividade antimicrobiana se baseia tanto no dano direto às membranas bacterianas através da liberação de íons hidroxila como também do seu alto pH que causa desnaturação protéica e dificulta a sobrevivência dos patógenos. Íons cloro são também capazes de quebrar ligações peptídicas, dissolvendo proteínas e liberando cloraminas que possuem atividade antibacteriana. O hipoclorito de sódio permanece hoje como uma das substâncias padrão ouro para irrigação endodôntica, muito embora seu efeito antimicrobiano não seja completamente eficaz (DARCEY *et al.,* 2015).

A clorexidina possui amplo espectro de ação contra bactérias Gram-positivas e Gram-negativas, além de também antifúngico. Sua atividade antimicrobiana resulta do rompimento da parede celular bacteriana (DARCEY *et al.,* 2015). Além disso, uma das maiores vantagens da clorexidina é sua substantividade. Devido seu poder de adesão às

paredes dentinarias, mantém suas propriedades antibacterianas por até 12 semanas. Todavia, seu uso permanece inferior ao hipoclorito de sódio já que não é capaz de dissolver matéria orgânica e sua taxa de penetração em biofilmes é menor que o hipoclorito (ROSSI-FEDELE *et al.,* 2011). Porém, diversos clínicos têm preferido o uso da clorexidina em virtude da toxicidade tecidual reduzida, quando comparada com o hipoclorito de sódio (PEMBERTON; GIBSON, 2012).

II.3.4.1 Terapias alternativas para descontaminação dos canais radiculares

Com o advento tecnológico das últimas décadas, novas modalidades e substâncias com atividade antimicrobiana têm sido desenvolvidas com a finalidade de aumentar a taxa de sucesso dos tratamentos endodônticos com um número menor de efeitos adversos ao paciente (VENKATESHBABU *et al.,* 2016).

Dentre essas variadas modalidades, três ganham destaque científico nos últimos anos. São eles: o uso de produtos naturais/herbais como irrigadores, aplicação de terapia fotodinâmica como método antimicrobiano, e aplicação de soluções a base de peptídeos antimicrobianos.

A procura por produtos mais biocompatíveis e menos agressivos ao tecido dentinário é o principal motivo do sucesso dos produtos naturais em Endodontia. Além de possuírem atividade antibacteriana comprovada, podem exercer efeitos benéficos ao tratamento endodôntico como substâncias anti-inflamatórios e antioxidantes. Dezenas de substâncias já foram testadas em ensaios *in vitro* e *in vivo* (VENKATESHBABU *et al.,* 2016). Dentre elas podemos citar o Aloe Vera, famoso por seu efeito anti-inflamatório potente que facilita o processo cicatricial e confere ação analgésica leve ao paciente (DAS *et al.,* 2011); o Própolis, em virtude do seu efeito antimicrobiano extremamente relevante, possuindo atividade *in vitro* comprovada contra cepas de *E. faecalis* (TYAGI *et al.,* 2013); e o Óleo de Copaíba, composto já incorporado a selantes dentinários com propriedade antibacteriana mediana, mas tolerância tecidual consideravelmente mais elevada que outros produtos (GARRIDO *et al.,* 2015).

Outra modalidade de tratamento que tem sido vastamente pesquisada na atualidade é o uso de Terapia Fotodinâmica Antimicrobiana (TFA) no combate à bactérias colonizadoras das estruturas dentais. Essa técnica é baseada no uso de lasers de baixa potência e que podem atuar em diferentes comprimentos de onda, com penetrabilidade moderada a baixa. O mecanismo de ação da TFA se dá através de um fotosensibilizador, uma fonte de luz

e oxigênio para morte bacteriana. Após a aplicação do fotosensibilizador no local a ser tratado, a fonte de luz, cujo comprimento de onda coincide com o pico de absorção do fotosensibilizador, estimula a produção de moléculas de oxigênio e radicais livres, resultando no dano celular bacteriano (PARKER, 2007; CHINIFORUSH *et al.,* 2015). As maiores vantagens da técnica é a sua invasividade mínima, não passível de resistência bacteriana e capacidade de repetição frequente. Contudo, o alcance da solução sensibilizante, bem como a taxa de penetração do laser são fatores que dificultam a perfeita ação antibacteriana (HAMBLIN; HASAN, 2004).

Por fim, os peptídeos antimicrobianos surgem como outro conjunto de moléculas com efeito antimicrobiano marcante e potencial uso comercial como soluções irrigadoras intracanais.

Muito embora abordemos mais aprofundadamente os peptídeos antimicrobianos no capítulo que se segue, é importante ressaltar que suas aplicações na área da Endodontia já é vislumbrada desde 2004 (TURNER; LOVE; LYONS, 2004). Desde então, essa ampla família de moléculas tem despertado interesse de vários pesquisadores ao redor do mundo por sua atividade antimicrobiana rápida, em baixas concentrações, amplo espectro de ação e, a depender da origem do peptídeo, excelente biocompatibilidade (DE FREITAS LIMA *et al.,* 2015). Na Tabela 2, adaptada de de Freitas Lima e colaboradores (2015), pode-se visualizar os peptídeos antimicrobianos estudados até o momento da publicação do trabalho e suas respectivas atividades antibacterianas contra diversas cepas envolvidas com lesões endodônticas.

Tabela 2: Lista dos peptídeos antimicrobianos testados contra microorganismos envolvidos com lesões endodônticas e suas respectivas concentrações inibitórias mínimas.

Peptídeo	Microorganismo	Concentração Inibitótia Mínima (µM)	Referência
Beta Defensina Humana (HBD) 3	*Fusobacterium nucleatum*	5	Song *et al*. (2009)
HBD3	*Enterococcus faecalis*	10	Song *et al*. (2009)
HBD3	*Tannerella forsythia*	>25	Ji *et al*. (2007)
HBD3	*Eikenella corrodens*	2	Ji *et al*. (2007)
HBD3	*Candida albicans*	10	Song *et al*. (2009)
SMAP29	*Fusobacterium nucleatum*	1	Weistroffer *et al*. (2008)
Defensina Neutrofílica Humana 1	*Prevotella intermedia*	2	Gursoy *et al*. (2013)
SMAP14A	*Peptostreptococcus micros*	2	Weistroffer *et al*. (2008)
LL-37	*Treponema denticola*	19	Rosen *et al*. (2012)
LL-37	*Enterococcus faecalis*	5	Thennarasu *et al*. (2010)
LL-37	*Porphyromonas gingivalis*	5	Thennarasu *et al*. (2010)
LL-37	*Tannerella forsythia*	>47	Ji *et al*. (2007)
LL-37	*Eikenella corrodens*	2	Ji *et al*. (2007)
HBD1	*Enterococcus faecalis*	10	Lee e Baek (2012)
HBD2	*Enterococcus faecalis*	5	Lee e Baek (2012)
HBD3	*Enterococcus faecalis*	1	Lee e Baek (2012)
HBD4	*Enterococcus faecalis*	3	Lee e Baek (2012)
Clavanina A	*Enterococcus faecalis*	1	Lee e Baek (2012)
Cecropina B	*Prevotella nigrescens*	25	Devine *et al*. (1999)
AR23	*Prevotella melaninogenica*	3	Urban *et al*. (2007)
Mellitina	*Candida albicans*	6	Urban *et al*. (2007)
SMAP28	*Actinomyces naeslundii*	1	Weistroffer *et al*. (2008)
SMAP28	*Actinobacillus actinomycetemcomitans*	1	Weistroffer *et al*. (2008)
SMAP28	*Actinomyces israelii*	1	Weistroffer *et al*. (2008)
Meta-fenileno-etileno (mPE)	*Actinomyces viscosus*	2	Beckloff *et al*. (2007)
mPE	*Actinobacillus actinomycetemcomitans*	1	Beckloff *et al*. (2007)
mPE	*Porphyromonas gingivalis*	4	Beckloff *et al*. (2007)
Chrysophsina 1	*Enterococcus faecalis*	3	Wang *et al*. (2012)
Chrysophsina 1	*Actinomyces naeslundii*	3	Wang *et al*. (2012)
Chrysophsina 1	*Streptococcus gordonii*	3	Wang *et al*. (2012)
Nisina	*Streptococcus gordonii*	150	Turner *et al*. (2004)
Nisina	*Enterococcus faecalis*	150	Turner *et al*. (2004)

Fonte: DE FREITAS LIMA *et al.,* 2015 com modificações.

II.4 PEPTÍDEOS ANTIMICROBIANOS

II.4.1 Aspectos Gerais

Mais de 7000 peptídeos naturais que desempenham funções biológicas nos seres humanos já foram identificados até a presente data. Tais moléculas desempenham papéis cruciais na fisiologia humana, incluindo a ação de hormônios, neurotransmissores, fatores de crescimento, ligantes de canais iônicos ou moléculas antimicrobianas do sistema imune inato (PADHI *et al*., 2014; BUCHWALD *et al*., 2014; GIORDANO *et al*., 2014; ROBINSON *et al*., 2014).

Entre os anos de 1920 e 1950, diversos compostos com propriedades antimicrobianas foram isolados de secreções de vários seres vivos como aracnídeos, anfíbios, vegetais e humanos (FLEMING, 1922; STUART; HARRIS, 1942). Tais compostos apresentavam atividade biológica em pequenas concentrações, além de exibir uma peculiar seletividade por bactérias Gram positivas e Gram negativas. Estes foram denominados de peptídeos antimicrobianos (PAMs) e descritos como moléculas capazes de inibir ou retardar o crescimento de microorganismos invasores, a fim de contribuir com os mecanismos naturais de imunidade adaptativa do hospedeiro (NAKATSUJI; GALLO, 2012).

Durante a última década, peptídeos antimicrobianos têm ganho um amplo campo de aplicações nas áreas biomédicas e biotecnológicas, além do crescimento em pesquisas visando os tais peptídeos como possíveis drogas terapêuticas. Alguns dos principais motivos do aumento no interesse em pesquisas com peptídeos antimicrobianos são: baixo custo e complexidade de produção; moléculas de pequeno tamanho molecular e com alta biocompatibilidade; e ação antimicrobiana em pequenas concentrações (FOSGERAU; HOFFMANN, 2015).

Segundo os mesmos autores, o mercado biotecnológico do peptídeos antimicrobianos se encontra em constante expansão. Estima-se que o mercado de síntese e comercialização de peptídeos, incluindo o de peptídeos antimicrobianos, tenha movimentado cerca de 14.1 bilhões de dólares em 2011 e estima-se, para 2018, um mercado de 25.4 bilhões de dólares (FOSGERAU; HOFFMANN, 2015).

Em um estudo de Wang e colaboradores, em 2015, foi realizado um levantamento bibliográfico das publicações sobre peptídeos antimicrobianos no qual se encontrou que, somente no ano de 2014, na base de dados Pubmed, houveram 7562 artigos científicos

publicados (aproximadamente 20 artigos por dia), dos quais 10% se tratavam de revisões de literatura.

Especificamente tratando-se do uso de peptídeos antimicrobianos sobre microorganismos orais, em estudo realizado por nosso grupo de pesquisa, em 2012, revisou-se a literatura científica com o intuito de melhor definir o uso de peptídeos antimicrobianos como ferramenta de controle de microorganismos patogênicos em cavidade oral. Neste estudo, foram avaliados artigos publicados e indexados nas bases de dados Pubmed durante o período de janeiro de 2002 a dezembro de 2011 (DA SILVA *et al*., 2012). Alguns resultados evidenciados em tal levantamento são abordados a seguir.

Na Tabela 3, evidencia-se o significativo crescimento no número de estudos que envolvem peptídeos antimicrobianos, os quais, quando relacionados a microorganismos orais, eram pouco avaliados nos oito anos iniciais do período aqui estudado. Entretanto, no último biênio, um grande salto na quantidade de publicações é constatado. Pode-se sugerir que o avanço nas técnicas de obtenção e purificação, além das constantes pesquisas sobre tais moléculas, são os principais fatores que ocasionaram este aumento quantitativo.

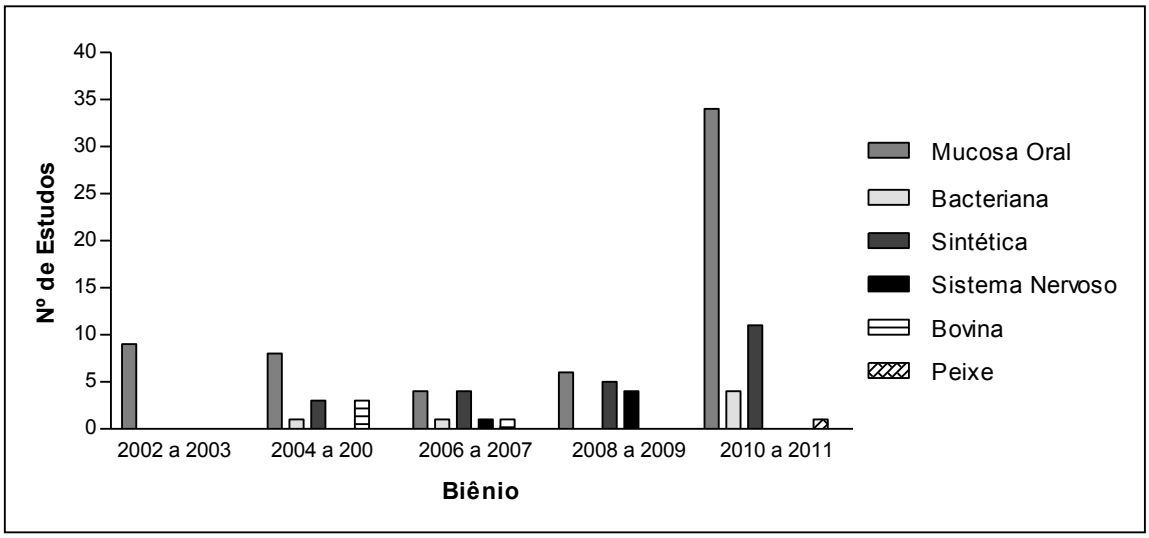
Tabela 3: Distribuição das publicações relacionadas a PAMs contra microorganismos orais de 2002 a 2011, por biênio.

	Valor absoluto (n)	**Valor relativo (%)**	**Valor relativo acumulativo (%)**
2002 a 2003	9	12.4	12.4
2004 a 2005	9	12.4	24.8
2006 a 2007	11	15	39.8
2008 a 2009	12	16.4	56.2
2010 a 2011	32	43.8	100
Total	108	100	

Fonte: DA SILVA *et al*., 2012.

Na Figura 18, retirada do mesmo estudo, pode-se verificar os principais tipos de peptídeos estudados de acordo com os biênios analisados. Nota-se uma predominância clara de estudos que utilizaram peptídeos oriundos da mucosa oral, provavelmente devido a já sabida participação dos PAMs no sistema imune inato das mucosas. Além desse grupo de peptídeos, os PAMs de origem sintética ocupam um lugar de crescente importância nos estudos microbiológicos, sendo a classe de peptídeos mais estudada logo depois dos peptídeos de cavidade oral.

Figura 18: Distribuição das publicações de acordo com a origem do PAM estudado, por biênio.



Fonte: DA SILVA *et al*., 2012.

V.1.2 Mecanismos de Ação Antimicrobiana

Os mecanismos de ação dos PAMs ainda não estão completamente esclarecidos. Todavia, algumas teorias sobre as formas de atuação dessas moléculas sobre a membrana celular e o metabolismo bacteriano têm sido sugeridas através de métodos como microscopia associada à fluoróforos, dicroísmo circular, espectrometria de massa e estudos com modelos de membrana (BOLINTINEANU; VIVCHARUK; KAZNESSIS, 2012; BROGDEN, 2005; PAULSEN *et al*., 2013). Dentre os principais mecanismos de atividade biocida sugeridos, têm-se os modelos de morte celular por via extracelular e os modelos por via intracelular (BROGDEN, 2005).

No que concerne aos modelos por via extracelular, os PAMs são inicialmente atraídos à superfície do microorganismo por interações eletrostáticas entre o peptídeo aniônico ou catiônico e estruturas na superfície celular. No caso de bactérias Gram-negativas, as principais interações ocorrem entre peptídeos catiônicos e a rede de cargas negativas presente no envelope bacteriano – fosfolipídios aniônicos e grupo fosfato nos lipopolisacarídeos. Enquanto que, em bactérias Gram-positivas, a principal relação é estabelecida pelos ácidos teicóicos da superfície celular (BOLINTINEANU; VIVCHARUK; KAZNESSIS, 2012).

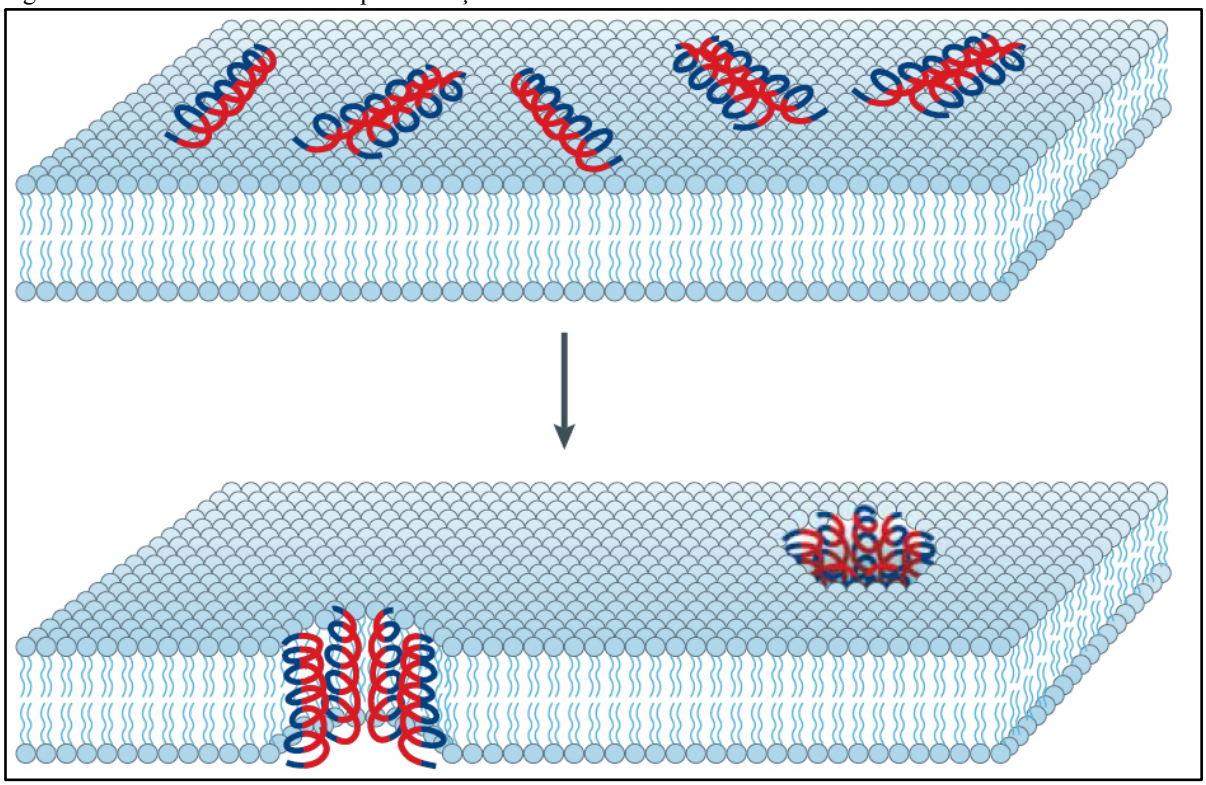
Após a atração inicial, os PAMs se ligam à superfície celular e iniciam a fase de ligação, fase esta responsável por realizar a travessia dos PAMs através da membrana externa (lipopolisacarídeos nas bactérias Gram-negativas e ácidos teicóicos e lipoteicóicos nas Gram-

positivas) e permitir que os PAMs interajam diretamente com a membrana citoplasmática bacteriana (BOLINTINEANU; VIVCHARUK; KAZNESSIS, 2012).

Em baixas proporções peptídeo/lipídio, os PAMs são ligados de forma paralela à bicamada lipídica. A medida que tal proporção aumenta, os peptídeos iniciam a sua orientação de forma perpendicular à membrana. Quando as proporções de peptídeo/lipídios estão elevadas, os PAMs iniciam sua penetração na membrana celular, o que gera a formação de poros transmembrana que, posteriormente, acarretam na morte celular por perda de líquido citoplasmático e rompimento membranar por micelização (PAULSEN *et al*., 2013).

Apesar de aparentemente simples, alguns modelos explicativos têm sido propostos para melhor entender a dinâmica da morte gerada por dano membranar. O modelo de barril ou "barrel-stave" sugere o arranjo de peptídeos em sentido transmembranar organizados em um formato de "barril", o que permite o fluxo de conteúdo intracelular para o meio externo (GKEKA; SARKISOV, 2010) (FIGURA 19).

Figura 19: Modelo "barrel-stave" para indução da morte bacteriana via PAM.

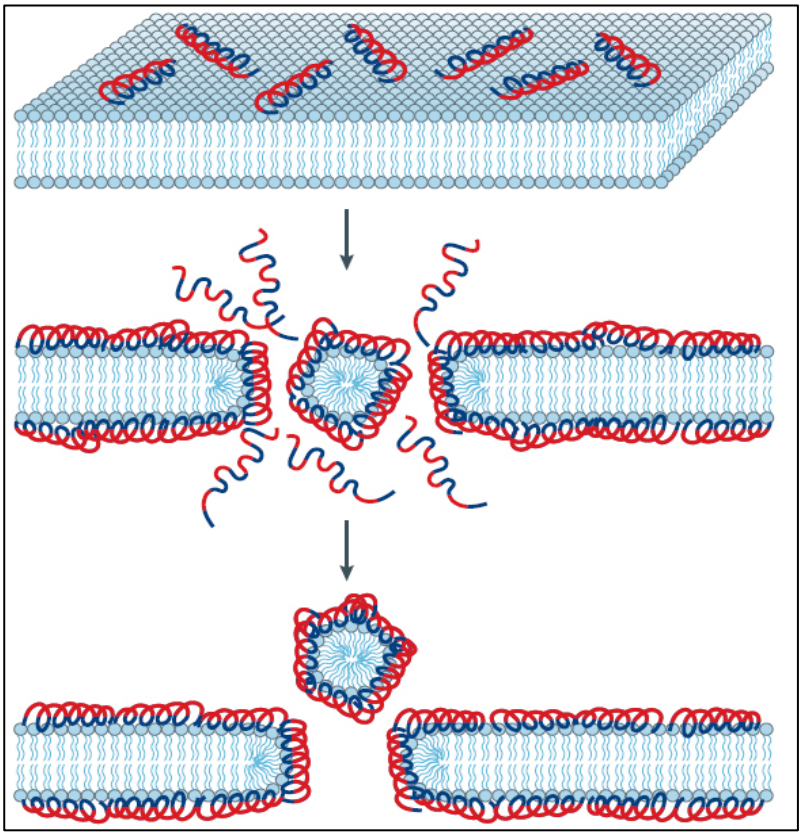


Fonte: BROGDEN, 2005.

Já o modelo de tapete ou "carpet model" ocorre um acúmulo de peptídeos na superfície da bicamada lipídica. Os pepetídeos são atraídos de forma eletrostática pelas extremidades aniônicas fosfolipídicas em diversos sítios recobrindo a membrana celular de uma maneira semelhante a um tapete. Em altas concentrações, os peptídeos são induzidos a

penetrar na membrana celular e romper a integridade da mesma de uma forma semelhante aos detergentes, levando eventualmente a formação de micelas. Em concentrações críticas, os peptídeos formam cavidades toroidais transientes na membrana, o que permite o acesso de mais peptídeos à membrana. Por fim, a membrana se desintegra e forma micelas após o rompimento da curvatura da bicamada (LEE *et al*., 2011) (FIGURA 20).

Figura 20: Modelo estilo "carpet model" para indução da morte bacteriana via PAM.

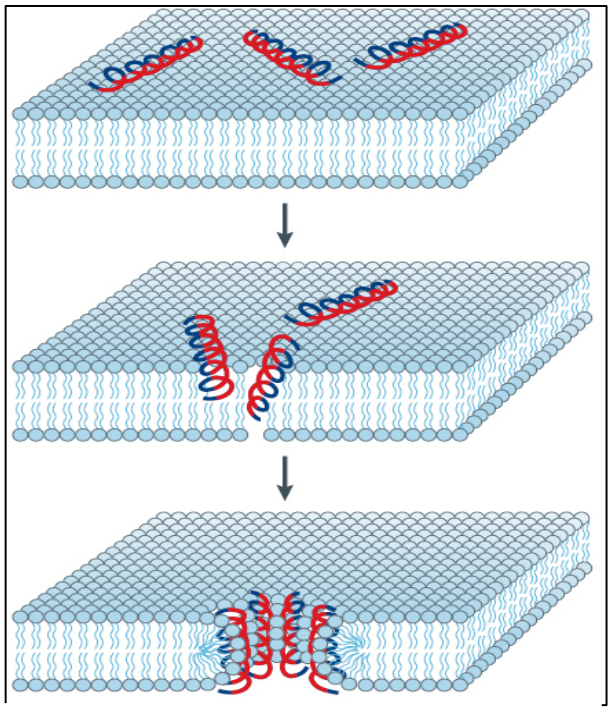


Fonte: BROGDEN, 2005.

Por fim, o modelo de poro-toroidal ou "toroidal-pore model" indica que a inserção das hélices dos PAMs na membrana induz a ligação de uma das monocamadas lipídicas à outra contraposta de forma contínua e bilateral, em que o núcleo aquoso é estabelecido por ambos, peptídeo inserido e extremidade lipídica. Na formação de poros toroidais, a face polar do peptídeo se associa com a superfície polar estabelecida pelas extremidades fosfolipídicas da membrana celular. Esse modelo se diferencia do modelo "barrel-stave" já que os peptídeos estão sempre associados às extremidades polares da monocamada, até mesmo quando organizadas perpendicularmente na bicamada celular (BOZELLI *et al*., 2011) (FIGURA 21).

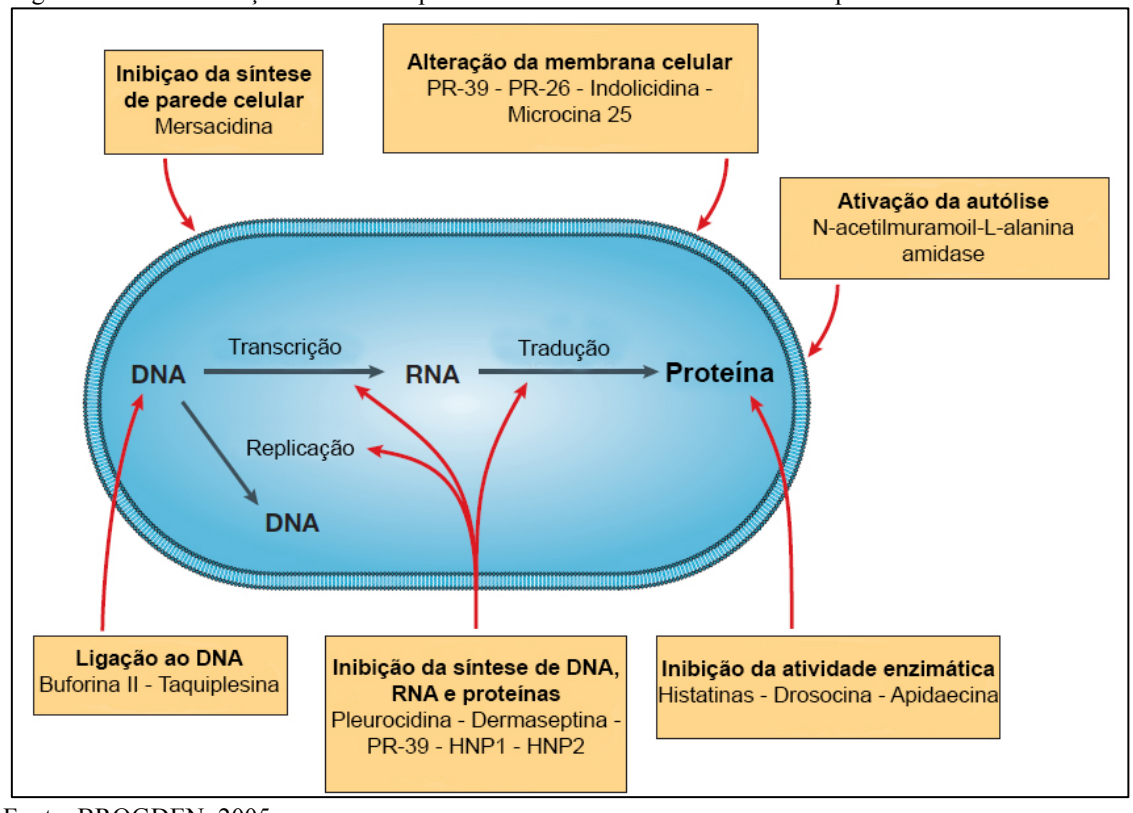
Muito embora a lise da membrana celular leve à morte bacteriana, um aumento no número de estudos que relatam a existência de outros métodos de morte celular, é notável. Pesquisas recentes revelaram a presença de alvos intracelulares importantes para a morte celular. Como exemplos de tais atividades têm-se a inibição da síntese de DNA, RNA e proteínas, geralmente relacionada com a pleurocidina, α-defensina-1 e α-defensina-2, e a inibição da atividade enzimática, associada com as histatinas (HALE; HANCOCK, 2007; MARR; MCGUIRE; MCMASTER, 2012; TEIXEIRA; FEIO; BASTOS, 2012) (FIGURA 22).

Figura 21: Modelo "Toroidal-pore" para indução da morte bacteriana via PAM.



Fonte: BROGDEN, 2005.

Figura 22: Modos de ação intracelular para atividade antimicrobiana mediada por PAMs.



Fonte: BROGDEN, 2005.

Não obstante às descrições de dano à membrana serem variáveis, elas estão, muito provavelmente, interrelacionadas. Brogden (2005) apontou que tais mecanismos não se apresentam de forma independente, mas sim correlacionadas e graduais. Porém, diversos fatores podem estar intimamente associados à efetividade e especificidade do peptídeo, tais como o seu tamanho, sequência de aminoácidos, carga, estrutura e conformação, hidrofobicidade e anfipaticidade (BROGDEN, 2005).

V.1.3 Peptídeos Antimicrobianos e sua Relação com a Imunidade

Durante as últimas duas décadas, lentamente, as diversas técnicas e estratégias utilizadas pelos vertebrados para lidar com microorganismos invasores têm sido reveladas (HANS; HANS, 2014). Nos tecidos, que primariamente entram em contato com tais agentes agressores, são secretados peptídeos antimicrobianos (PAMs), moléculas pequenas derivadas de proteínas precursoras. Suas capacidades catiônica e anfipática permitem que tais moléculas atuem de maneira vasta objetivando a morte de invasores (HEMSHEKHAR; ANAPARTI; MOOKHERJEE, 2016).

Certos leucócitos de mamíferos, denominados de granulócitos, possuem grânulos em seu citoplasma que armazenam grande quantidade de precursores de PAMs (OUELLETTE, 2004). Esses precursores são lisados em PAMs ativos quando secretados em fagossomas. Dessa forma, quando microorganismos forem fagocitados, os PAMs serão os principais responsáveis por causar a morte direta do patógeno (DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006).

Entretanto, apesar de sua nomenclatura sugestiva, acredita-se que os peptídeos antimicrobianos não detenham apenas a capacidade de morte de microorganismos. Isso se justifica quando analisam-se peptídeos antimicrobianos no soro sanguíneo ou em ambiente tecidual. Tais moléculas perdem a sua capacidade microbiocida, contudo, aparentam desempenhar funções imunomodulatórias em uma complexa rede entre a imunidade inata e adquirida (HANCOCK, 2001; ELSBACH, 2003).

Em mamíferos, pelo menos dois grupos distintos de peptídeos antimicrobianos e seus respectivos precursores podem ser encontrados. As Defensinas, grupo de moléculas com alta capacidade antimicrobiana e com funções bem estabelecidas na imunomodulação, processo cicatricial e combate ao câncer; e as Catelicidinas, grupo de precursores cujos peptídeos derivados desempenham uma gama de atividades biológicas fundamentais na manutenção da homeostasia dos tecidos mucosos (HEMSHEKHAR; ANAPARTI;

MOOKHERJEE, 2016). Alguns pesquisadores, como De Smet e Contreras (2005), ainda destacam as Histatinas como um terceiro grupo de peptídeos antimicrobianos com vasta importância na regulação da imunidade e combate à agentes externos. Em virtude do objetivo principal do presente trabalho, abordaremos em maior profundidade a família das Catelicidinas.

V.1.3.1 Catelicidinas

As catelicidinas formam uma classe distinta de proteínas precursoras presentes na imunidade inata de todos os mamíferos. De forma semelhante às defensinas, agem liberando peptídeos antimicrobianos ativos por quebra proteolítica. Contudo, suas características estruturais diferem claramente das defensinas (OKUMURA, 2011; VANDAMME *et al*., 2012).

A descoberta da família das catelicidinas iniciou-se após o isolamento de um peptídeo antimicrobiano denominado de Bac5 presente em neutrófilos bovinos, e a descoberta que tal peptídeo, na verdade, era oriundo de um precursor protéico. Análises genéticas permitiram a identificação de um grupo de genes responsável pela codificação de toda a família das catelicidinas (VANDAMME *et al*., 2012).

As proteínas do grupo catelicidina são caracterizadas por um domínio N-terminal altamente conservado de aproximadamente 100 resíduos de aminoácidos. Além disso, possuem um domínio de 14 kDa semelhante à catelina seguida por um domínio peptídeo sinal em seu N-terminal (com aproximadamente 30 aminoácidos de comprimento), e uma região com capacidade antimicrobiana em sua região C-terminal (OKUMURA, 2011).

O produto primário da tradução das catelicidinas é denominado pré-proteína, já que possui um peptídeo sinal como um prelúdio para a proteína em si. Esse peptídeo sinal é quebrado no momento em que o mesmo completa sua função de direcionamento aos grânulos de armazenamento ou ao exterior da célula. A formação de duas pontes dissulfeto finaliza o processamento pós-traducional da catelicidina. Essa é então referida como uma pró-proteína, pois não representa a forma ativa e sim a de armazenamento. Somente após sua quebra em domínio catelina e domínio antimicrobiano que suas atividades poderão ser completamente desempenhadas (HANCOCK, 2001).

Ao contrário do domínio catelina altamente conservado das catelicidinas, os peptídeos antimicrobianos derivados podem possuir diferentes estruturas primárias e secundárias a depender da forma de quebra proteolítica ou do ambiente em que tais moléculas

se encontram. Estudo já demonstrou que peptídeos em α-hélice ricos em prolina, arginina ou triptofano (indolicidina) e peptídeos ligados por cisteínas (docapeptídeos e integrinas) são derivados antimicrobianos das catelicidinas. (OKUMURA, 2011)

Dentre os diversos domínios peptídicos antimicrobianos conhecidos, a classe de peptídeos em α-hélice LL-37 é uma das mais amplamente estudadas e conhecidas na atualidade (VANDAMME *et al.,* 2012).

LL-37 e seu precursor protéico, hCAP18, são encontrados em diferentes concentrações nos mais diversos tecidos e fluidos humanos. Tal fato está intimamente relacionada tanto a sua atividade como peptídeo antibiótico como também molécula de sinalização (THENNARASU, *et al.,* 2010).

LL-37 foi inicialmente descrito em leucócitos, mas foi brevemente descoberto em uma ampla variedade de tecidos e células. Uma das principais células produtoras de LL-37 são os granulócitos que acumulam este peptídeo a medida que maturam e, quando em estado de ativação, liberam suas formas ativas para o meio externo (HEMSHEKHAR; ANAPARTI; MOOKHERJEE, 2016).

Atualmente sabe-se que taxa de expressão de LL-37 nas diversas células do corpo está diretamente relacionada com o desenvolvimento de diversas doenças ou ao aumento de susceptibilidade do indivíduo a contrair infecções (DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006).

Em geral, a sobre regulação de LL-37 acontece mais frequentemente do que a subregulação, indicando o suporte de LL-37 no combate a infecções. A produção de toxinas pelas bactérias que colonizam o trato respiratório é capaz de causar um aumento significativo na produção de LL-37 pelas células epiteliais locais. Evento semelhante também acontece em células epiteliais mamárias e de colo retal (HEMSHEKHAR; ANAPARTI; MOOKHERJEE, 2016).

Ao contrário, a redução de LL-37 também pode estar relacionada a determinados casos, como, por exemplo, em pacientes com úlceras crônicas e dermatites recorrentes. Além disso, foi relatado que, em alguns pacientes com infecções intestinais frequentes, os níveis de LL-37 produzidos pelo tecido mucoso são comparativamente reduzidos, fato este que aumenta a taxa de proliferação bacteriana local (DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006).

Contudo, o LL-37 foi inicialmente reconhecido por sua capacidade antimicrobiana, exibindo atividades bactericida, fungicida e viruscida. Quanto ao seu efeito antibacteriano, LL-37 exibe um amplo espectro de ação contra bactérias Gram-positivas e

Gram-negativas de diferentes gêneros (DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006). Na tabela 4 estão listados resultados de atividade de LL-37 contra algumas bactérias estudadas até então com suas respectivas concentrações inibitórias mínimas.

A atividade fungicida e viruscida do LL-37, muito embora comprovada, ainda é pouco abordada. Somente um estudo realizado por Larrick, Hirata e Zhong (1995) comprovou atividade inibitória de LL-37 frente a cepas de *Candida albicans*. Já para o seu efeito contra vírus, dois estudos foram realizados nos quais este peptídeo foi capaz de inativar fracamente os vírus da herpes simples e da varíola (YASIN et al., 2000; HOWELL et al., 2004).

Tabela 4: Resumo dos resultados publicados sobre a atividade antimicrobiana do peptídeo LL-37.

Microorganismo	**Atividade de LL-37**
Bactérias Gram-positivas	
Streptococcus	
Group A	1–16 Mm
Group B	≥32 μM
Group C	16 μM
Staphylococcus aureus	>32 μM
Enterococcus faecalis	30 μg/ml
Staphylococcus epidermidis	7.6 μg/ml
Listeria monocytogenes	1.5 μg/ml
Enterococcus faecium	0.7 μg/ml
Lactobacillus acidophilus	19 μM
Bacillus subtilis	2.7 μg/ml
Bacillus megaterium	0.2 μM
Bactérias Gram-negativas	
Escherichia coli	>32 μM
Pseudomonas aeruginosa	16 μg/ml
Actinobacillus actinomycetemcomitans	10 μg/ml
Salmonella typhimurium	3.5 μg/ml
Salmonella minnesota	0.2 μg/ml
Burkholderia cepacia	79 μg/ml
Capnocytophaga ochracea	11 μg/ml
Klebsiella pneumoniae	4.2 μg/ml

Proteus mirabilis	5.7 μg/ml
Stenotrophomonas altophilia	1.9 μg/ml
Proteus vulgaris	2.5 μg/ml
Capnocytophaga sputigena	7.5 μg/ml
Capnocytophaga gingivalis	9 μg/ml
Salmonella serovar Dublin	2.8 – 6.0 μM
Espiroquetas	
Leptospira interrogans	225 μg/ml
Borrelia spp.	450 μg/ml
Treponema pallidum	450 μg/ml
Fungos	
Candida albicans	>20 μg/ml

Fonte: DÜRR; SUDHEENDRA; RAMAMOORTHY, 2006

Baseando-se em estudos por Ressonância Magnética Nuclear, Wang e colaboradores, em 2012, identificaram a principal porção antimicrobiana do peptídeo LL-37, sendo esta compreendida pelos aminoácidos 17-32. O peptídeo correspondente, GF-17, foi ativo contra cepas de Staphylococcus aureus e Escherichia coli. Contudo, em estudos posteriores, utilizando-se de técnicas de síntese molecular e análise físico-química, os mesmos autores evidenciaram que a porção antimicrobiana ativa poderia ser ainda menor e a representaram pelos aminoácidos 18-29 do peptídeo humano LL-37. Dessa forma, o peptídeo KR-12 (KRIVQRIKDFLR-NH_2) foi então descoberto como um dos menores peptídeos antibacterianos até o momento (MISHRA *et al.,* 2013).

Poucos estudos acerca da atividade antimicrobiana do peptídeo KR-12 foram realizados até o momento. Contudo, algumas características desse peptídeo já foram elucidadas que o tornam um excelente modelo para construção de peptídeos análogos. Um das características é sua baixa citotoxicidade, fato este que o destaca de outros PAMs que são danosos à membranas celulares. Outro ponto é sua não precipitação em meio de cultura, o que facilita a realização de testes antimicrobianos *in vitro* sem interferências (WANG et al., 2012).

V.1.4 Peptídeos Sintéticos

Os peptídeos antimicrobianos naturais possuem uma série de características que os colocam em posição interessante para se tornarem comercialmente viáveis. Contudo, nos últimos anos, com o avanço na tecnologia de síntese molecular e de estudos mais aprofundados sobre os sítios responsáveis pela atividade antimicrobiana, o desenvolvimento em larga escala de peptídeos sintéticos melhorados tem atraído atenção de industrias farmacêuticas ao redor do mundo (WANG *et al.,* 2015).

A maioria dos peptídeos naturais possuem sequências longas, fato este que os tornam mais instáveis e aumenta o custo de produção. A síntese de novos peptídeos ativos curtos oferecem benefícios relacionados à facilidade de composição molecular, simples de sintetizar e modificar sua sequência visando aumento de estabilidade, diminuição de toxicidade e imunogenicidade (RAMESH *et al.,* 2016).

A síntese de peptídeos curtos não é novidade no campo da microbiologia. Em 1992, Blondelle e Houghten estudaram a atividade antimicrobiana de peptídeos sintéticos contendo somente unidades dos aminoácidos Leucina e Lisina contra cepas bacterianas Gram-positivas e Gram-negativas.

Anos depois, em 2005, Deslouches e colaboradores perceberam que a substituição de um aminoácido central por um triptofano pode modificar o perfil antimicrobiano de alguns peptídeos.

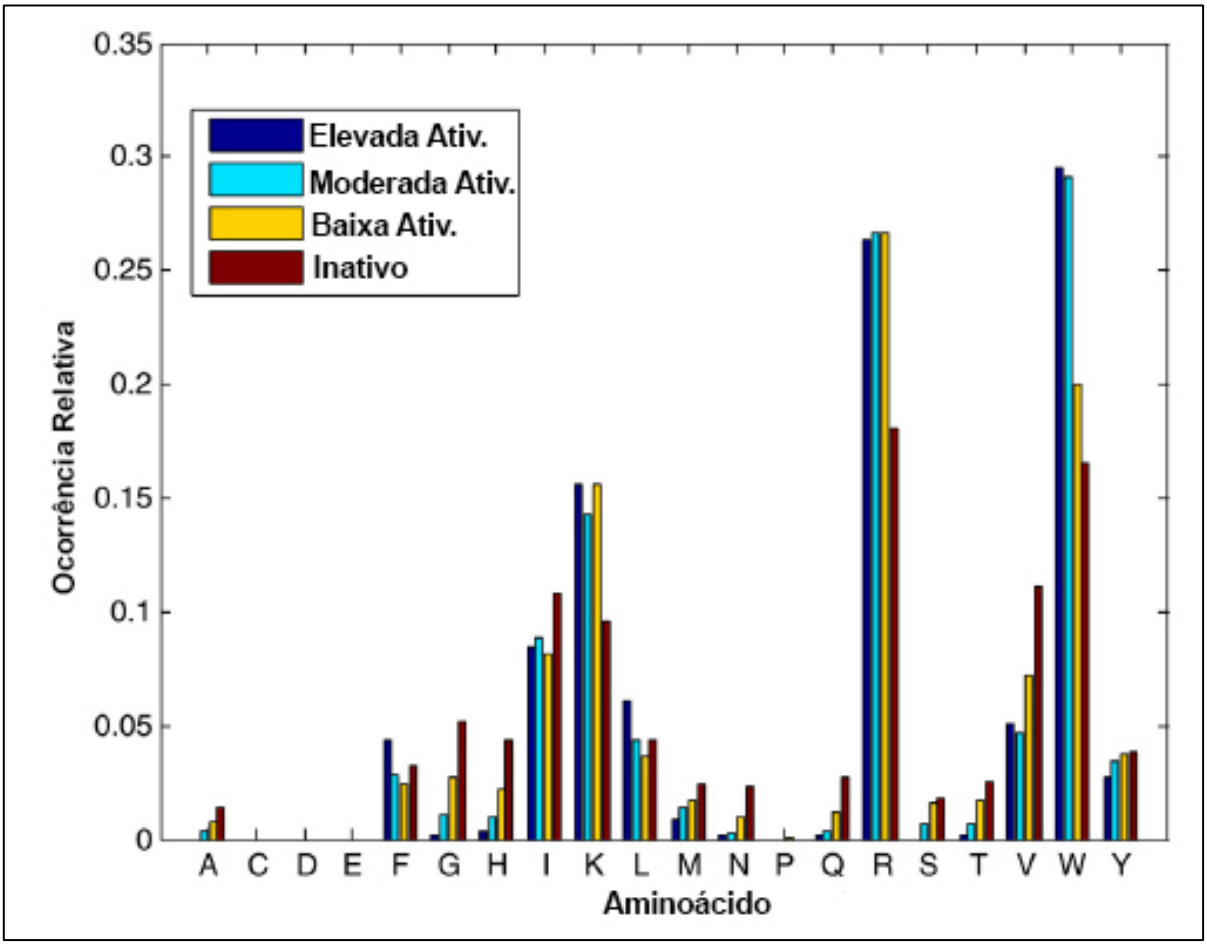
Mais recentemente, Mikut e colaboradores, em 2016, analizaram as influências que alterações de posição do aminoácido na sequência primária, bem como sua extensão, causam na atividade antimicrobiana de mais de 3000 peptídeos desenhados e sintetizados pelos próprios autores baseados em diversos bancos de dados.

Nesse mesmo estudo, os autores classificaram as atividades antimicrobianas em: Elevada, Moderada, Fraca e Inativa. Como resultados, verificaram que para peptídeos com elevada atividade antimicrobiana, existe uma preferência por aminoácidos hidrofóbicos (W, I, L, V e Y) na porção central do peptídeo. Além disso, perceberam que o equilíbrio entre a carga positiva do peptídeo e sua hidrofobicidade é fundamental para sua atividade e que a presença do aminoácido Triptofano se mostrou importante em todos os peptídeos de alta atividade.

Por fim, foi percebido que, em peptídeos com moderada e alta atividades, o aminoácido Lisina foi relatado em abundância principalmente nas porções terminais do peptídeo, indicando uma possível influência e participação dessa região na atividade antimicrobiana dos peptídeos (MIKUT *et al.,* 2015).

Na Figura 23 pode-se notar a ocorrência relativa de aminoácidos presentes nos peptídeos sintéticos avaliados de acordo com seu grau de atividade contra as cepas bacterianas testadas.

Figura 23: Ocorrência relativa de aminoácidos em diversos peptídeos sintéticos de acordo com seu grau de atividade antimicrobiana.



Fonte: Mikut et al., 2015 com modificações.

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Capítulo III – Artigo Científico I

Synthesis of a novel antimicrobial peptide derived from KR-12 with activity on planktonic cells and biofilms of Streptococcus mutans.

Proposta de Submissão: Biofouling (Fator de Impacto: 3.000)

Research Article

Synthesis of a novel antimicrobial peptide derived from KR-12 with activity on planktonic cells and biofilms of Streptococcus mutans

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Abstract: The aims of this study were to describe the synthesis of a novel synthetic peptide based on the primary structure of KR-12 peptide and evaluate its antimicrobial and antibiofilm activity on *Streptococcus mutans* strains. The methods used to evaluate antimicrobial activity include the following: determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in microtiter plates for growth in suspension and quantification of biomass by crystal violet staining and counting of colony forming units and visualization by scanning electron microscopy for biofilm growth. The tested peptide demonstrated a remarkable antimicrobial effect, interfering on the planktonic and biofilm growth of all strains tested, even at low concentrations. Thus, the peptide [W^7]*KR12*-KAEK is an important source for potential antimicrobial agents, especially for the control and prevention of microbial biofilms, which is one of the most important factors in cariogenic processes.

Keywords: Antimicrobial Peptide; *Streptococcus mutans*; Biofilm; Dental Caries.

Introduction

The term dental caries was initially used in the literature around 1634 with the sole purpose of describing the orifices generated in the dental structures, which were responsible for severe pain and subsequent tooth loss (Bowen 2015). However, at that time, no knowledge about the pathophysiological process of the disease has been elucidated. Currently, it is known that dental caries is considered as a multifactorial disease, including a number of causative and modifiers factors, both endogenous and/or exogenous (Hanioka et al. 2011; Kalesinskas et al. 2014).

Among the factors related to the development of dental caries and other oral diseases, there is the presence of dental biofilms. Biofilms are defined as microbial communities adhered to each other covered with a polymeric extracellular matrix produced both by microorganisms themselves, but also compounds derived from the host (Bjarnsholt et al. 2013). In the case of cariogenic biofilms, they still have high acidogenic ability, responsible for the decrease in local pH and demineralization of tooth tissue (Bowen & Koo 2011).

One of the main microorganisms responsible for the beginning of the demineralization process and consolidation of the acidic pH in the cariogenic biofilm is *Streptococcus mutans*. This bacteria is a Gram-positive cocci, natural colonizer the oral cavity, first identified by Clarke (1924). Currently, it is known that *S. mutans*, under favorable environmental conditions, can proliferate excessively in the oral cavity and use its diverse gene repertoire in the metabolism of carbohydrates and the production of acids that contribute in the pH drop locally (Salli et al. 2016)

Due to the high incidence of dental caries around the globe and to the preventive means highly dependent of individual collaboration, new therapies have been researched to control the pathogenic oral microflora and thus reduce the incidence of such lesions (Walsh et al. 2015).

One of the therapies that have received great visibility currently aims to use small molecules with high antibacterial activity and low cytotoxic activity, the antimicrobial peptides (AMPs) (Silva et al. 2012). AMPs are a heterogeneous group of molecules produced by a wide range of tissues and cells in

many invertebrates, plants and animals, usually acting on innate defense system due to its antimicrobial characteristics against a wide variety of microorganisms (Batoni et al. 2011; Tao et al. 2011).

Our research group conducted recently a survey of the literature that revealed the high impact of these molecules in the control of microorganisms of the oral cavity. Among the different types and groups of AMPs, the peptides produced in the oral cavity by the immune system were highly studied because of their greater antimicrobial activity and low aggression to oral mucosal tissues (Silva et al. 2012).

The antimicrobial peptide KR-12 is considered the lowest natural active peptide known, corresponding to amino acids 18-29 of the human cathelicidin peptide LL-37 (Wang 2008). Due to its small size, low toxicity and high solubility in culture media, this peptide has served as template in the design of new synthetic antimicrobial molecules (Mishra et al. 2013).

Thus, the objective of this study was to describe the process of synthesis of a novel synthetic peptide based on the primary structure of KR-12 and evaluate its antimicrobial and antibiofilm activity against three different strains of *Streptococcus mutans.*

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains involved in the cariogenic process, *Streptococcus mutans* ATCC25175, *Streptococcus mutans* UA 159, and *Streptococcus mutans* UA 130 were grown in Brain Heart Infusion Agar (BHIA, Difco, Himedia, India) for 24 h 37°C and 5% CO_2. After growth on Agar medium, isolated colonies were removed an inoculated into 5 ml of sterile medium BHI broth supplemented with 1% sucrose (BHIs) under the same conditions. Prior to assays, bacterial cells were adjusted to concentration of 10^6 CFU ml^{-1} in BHIs for all experiments of antimicrobial and antibiofilm activity.

Peptide synthesis, purification and preparation

The peptide [W^7]*KR12*-KAEK (KRIVQRWKDFLRKAEK-NH_2) were synthesized using a Protein PS-3 synthesizer by solid phase peptide synthesis (Merrifield 1963) using standard 9-fluorenylmethyloxycarbonyl (Fmoc) protocols on Fmoc-Arg(Pbf). The amino acids were coupled at a four-fold excess using O-Benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate (HBTU)/N-methylmorpholine (NMM) (v/v) with N,N-dimethylformamide (DMF). The α-amino group deprotection step was performed in 20% piperidine/DMF for 1 and 20 min. Cleavage of the peptide from the resin and removal of the side chain protecting groups were simultaneously performed with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropyl silane for 2 h. After this procedure, the crude peptide was precipitated with anhydrous ethyl ether, separated from soluble non-peptide material by centrifugation, extracted into 5% solvent B (0.036% (v/v) TFA/acetonitrile) and 95% solvent A (0.045% (v/v) TFA/H_2O) and lyophilized. The crude peptide was purified by semi-preparative HPLC on a Beckman System Gold using a reversed-phase C18 column with a linear gradient of 5–45% solvent B for 90 min. The flow rate was 5 ml min^{-1}. Ultraviolet (UV) detection was carried out at 220 nm. The peptide homogeneity was checked by analytical HPLC on a Shimadzu

system, using solvents A and B with a linear gradient of 5–95% (v/v) solvent B for 30 min, at a flow rate of 1.0 ml min^{-1} and UV detection at 220 nm. The identity of the peptide was confirmed by mass spectrometry in positive-ion mode ESI on a Bruker model apparatus.

The peptide was fully solubilized in sterile deionized water with 0.1% acetic acid (CH_3COOH) at a concentration of 500 μg ml^{-1} prior to use and stored in a freezer at -20ºC.

Antimicrobial activity assay

The antimicrobial activity of [W^7]*KR12*-KAEK was assessed by the microdilution test in 96-well "U" bottom microtiter plates. The plates were prepared with sterile BHIs containing the peptide [W^7]*KR12*-KAEK at a concentration of 500 μg ml^{-1}. Then, two-fold serial dilutions were made to achieve varying concentrations (7.8 to 500 μg ml^{-1}) at a final volume of 100 μl. Next, 100 μl of cells (2 x 10^6 CFU ml^{-1}) was added to each well to yield a final volume of 200 μl. Chlorhexidine gluconate (0.12%) and BHIs sterile culture medium were added to separate wells as the positive and negative controls, respectively.

The plates were incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the turbidity of each well was measured using a spectrophotometer (SpectraMax® I3) at 620 nm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of inhibiting bacterial growth visually.

To determine the minimum bactericidal concentration (MBC), Petri dishes with BHI agar were inoculated with 10 μl from the wells that showed no visible microbial growth. The MBC was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of completely inhibiting microbial growth on the Petri dishes.

Antibiofilm activity assay

The assays of biofilm formation were performed according to the methodology described by Stepanovic (2000) in 96-well "flat" bottom microtiter plates with some modifications. The plates were prepared in the same manner as in the antimicrobial activity test. However, two plates were prepared for each test, one for the quantification of biomass (crystal violet) and one for counting the CFU (Colony Forming Units) adhered to the plate.

Quantification of biomass

After 24 hours of incubation at 37°C, the plates were washed three times with sterile distilled water, and then 200 μl of methyl alcohol P.A. was added to fix adhered cells. After the removal of methanol, 200 μl of 1% crystal violet was added for 5 minutes. This procedure was performed to allow an indirect quantification of biofilm formation by staining with crystal violet. Then, the washing procedure was repeated and the plate placed at room temperature for drying. To dissolve the crystal violet bound to the biofilm, 200 μl of 33% acetic acid were added to wells and the absorbance of each well was measured with a spectrophotometer (SpectraMax® I3) at 595 nm.

Count of Colony Forming Units (CFU)

After incubation for 24 hours at 37°C, the plates were washed three times with sterile distilled water to remove weakly attached cells. Next, 200 μl of sterile 0.9% NaCl solution was added to each well of the plate, and the plate was then subjected to an ultrasonic bath (Sonicor/SC-52) for 6 minutes to release the cells from the formed biofilm. The volume of five wells was removed with an up-down movement and collected into a sterile 2.0 ml microtube to a final volume of 1 ml. Ten-fold serial dilutions of the cell suspension were performed for subsequent plating. Then, the plates were incubated at 37°C with 5% CO_2 for 24 hours. The number of colonies grown on each plate were counted and converted to units of CFU ml^{-1} (Log_{10}).

Pre-formed biofilm assay

To evaluate the activity of [W^7]*KR12*-KAEK on bacterial mature biofilms, 200 μl of cells suspension (10^6 cells ml^{-1}) were added to each well and incubated for 24 h at 37°C with 5% CO_2. After biofilm development the wells were washed twice with 200 μl of sterile distilled water for removal of non-adhered cells and an aliquot of 200 μl of the peptide solubilized in BHIs (at concentrations ranging from 7.8 to 500 μg ml^{-1}) was added to the wells. Chlorhexidine gluconate (0.12%) and BHIs sterile culture medium were added to separate wells as the positive and negative controls, respectively. The plates were incubated for 24 h at 37°C in 5% CO_2. After 24 h, the medium was removed and each well was the washed twice with 200 μl of sterile saline solution. The effect of [W^7]*KR12*-KAEK on pre-formed biofilms was evaluated by biomass quantification and count of colony forming units as described above.

Antibiofilm analysis of [W^7]KR12-KAEK in scanning electron microscopy

The polystyrene plates were set up and incubated as described above for tests in preformed biofilms but testing only de peptide [W^7]*KR12*-KAEK at a concentration of 500 μg ml^{-1} and the negative control against the strain *S. mutans* UA130.

The plates were removed from incubation and weakly adherent cells were removed by washing with sterile ultrapure water, and then allowed to dry at ambient temperature. Wells containing biofilms were dehydrated sequentially with ethanol (70% ethanol for 10 minutes, 95% for 10 minutes and 100% for 20 minutes) and allowed to dry at ambient temperature. After drying, they were kept in desiccator until analysis. Before observation, cells were mounted on aluminum holders and covered with gold particles and then observed under the scanning electron microscope S-360 (Leo, Cambridge, USA).

Circular dicroism spectra

Circular dichroism spectra were obtained between 190 and 250 nm with a JASCO J-815 CD spectrophotometer (Japan) on nitrogen flush in 1 mm path length quartz cuvettes at room temperature. The peptide concentration was 85 μmol l^{-1}. To investigate the conformational changes by membrane environments, a solution containing 10 mmol l^{-1} of lysophosphatidylcholine (LPC) was used. CD spectra were recorded as an average of six scans that were obtained in millidegrees and converted to molar ellipticity [θ] (in deg cm^2 $dmol^{-1}$) Curves were smoothed to eliminate instrument noise.

Statistical analysis

All experiments were performed in triplicate, and the results were entered into Microsoft Excel (Version 2010 for Windows) and later analyzed with GraphPad Prism software (version 5.0 for Windows). To analyze the significant differences between groups, one-way ANOVA was conducted with a Bonferroni post-test, and $p<0.05$ was considered significant.

Results

Peptide synthesis

We have designed a new peptide analogue from KR12 called [W^7]KR12-KAEK. This peptide contains a Trp residue in position 7. This could allow more interaction between the peptide and membrane, improving the biological activity (Persson et al. 1998; Yau et al. 1998). In addition, the sequence KAEK was added at C-terminus position of KR12 to evaluate the increase of size chain of peptide and the amphipathicity. This modification also increased the charge of peptide. The peptide KR12 has charge +5 on physiological pH and the analogue +6. The mainly physicochemical properties of KR12 and analogue are shown in Table 1.

Table 1: Physicochemical properties of KR12 and [W7]KR12-KAEK.

Peptide/Property	**KR12**	**[W^7]*KR12*-KAEK**
Net charge	+5	+6
Charge density	2.4	2.6
Hydrophobicity*	11.8	12.0
% Hydrophobic residues	41.2	37.5
Hydrophobic Moment Vectors**	45.2	53.3

*RP-HPLC Retention time **Angle between HM vector and z-axis.

The Schiffer–Edmundson helical wheel diagram (Figure 1) of KR12 and [W^7]KR12-KAEK demonstrate that the peptide have an amphipathic alpha-helix conformation with hydrophilic and hydrophobic residues on opposite sides forming an amphipathic conformation. This structure was increased with the modifications in the analogue peptide.

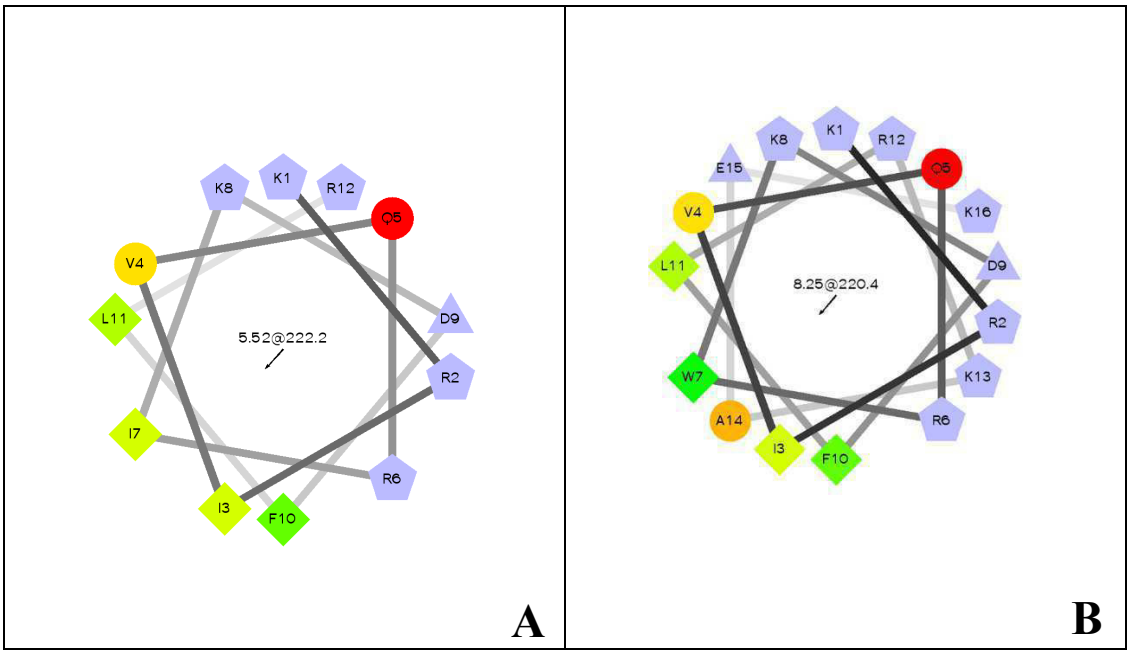

Figure 1: Schiffer–Edmundson helical wheel diagram demonstrating probable amphipathic a-helical conformation of KR12 (A) and [W^7]KR12-KAEK (B). Hydrophilic residues as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons.
(http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?sequence=ABCDEFGHIJLKMNOP&submit=Submit)

To confirm the amphipathic alpha-helical structure of [W^7]KR12-KAEK, the secondary structures were obtained by CD in PBS solution and LPC. The results presented in Figure 2 show that the analogue peptide has a random coil structure in buffer solution. On the other hand, in the presence of LPC, the peptide acquired a well-defined helical structure, with double minima at 207 and 222 nm and a maximum at around 195 nm. These results were similar to obtained for KR12 (Mishra et al. 2013). In this study, the KR-12 did not form helical structure in PBS buffer but became helical in the presence of lipid with double minima at 207 and 222 nm and a maximum at around 195 nm. However is important to note that in PBS the KR12 showed a minima around 222 nm that indicates the presence of small quantity of helical structure (Figure 3). It was not observed to the analogue peptide. This observation shows that probably the [W^7]KR12-KAEK has smaller tendency of self-associate in solution. The helical content in solution under physiological conditions has been described as important feature for biological activity (Ulaeto et al. 2016). These differences in physicochemical properties could explain the different biological activity between the KR12 and its analogue.

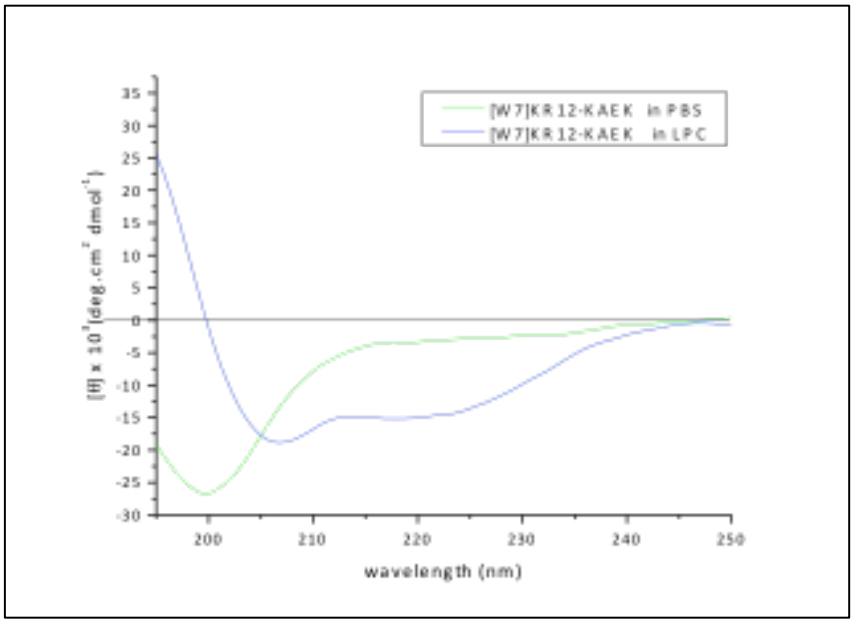

Figure 2: CD spectra of [W7]KR12-KAEK in PBS (pH: 7.2) and LPC (10 mmol l^{-1}) micelles. The peptide concentration was 30 µmol l^{-1}.

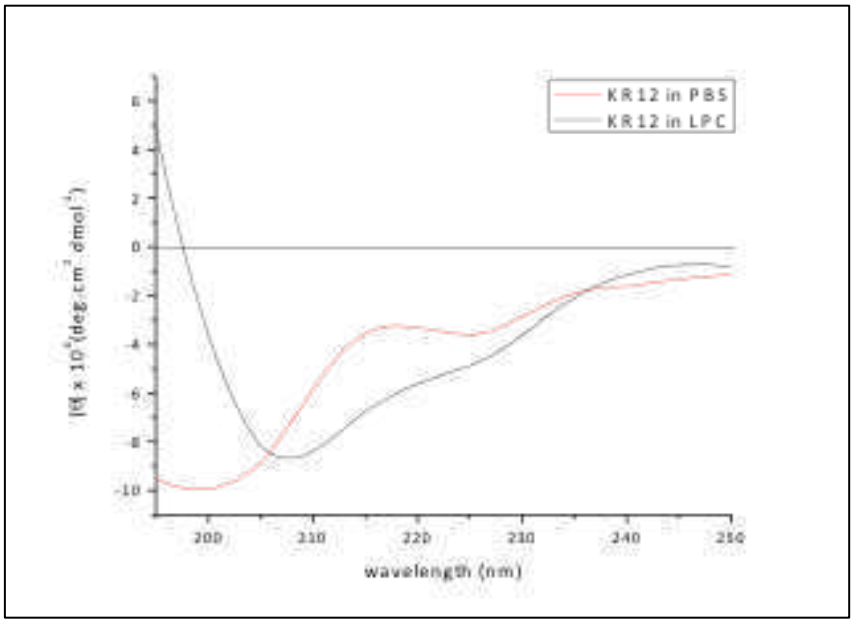

Figure 3: CD spectra of KR12 in PBS (pH: 7.2) and LPC (10 mmol l^{-1}) micelles. The peptide concentration was 30 µmol l^{-1}.

Antimicrobial activity

Concerning the antimicrobial activity, the two peptides showed considerably different activities across the tested bacterial strains.

In Table 2, it can be noticed that the peptide KR12 did not show significant antimicrobial activity through MIC and MBC values not set out until the concentration of 500 µg ml^{-1}. However, the synthetic peptide [W^7]*KR12*-KAEK demonstrated different antimicrobial activities for the various strains tested (Table 2). The MIC values ranged from 7.8 to 31.25 µg ml^{-1}, while the MBC values ranged from 15.6 to 62.5 µg ml^{-1}.

Table 2: *In vitro* susceptibility of oral microorganisms tested against the peptide KR12 and its analogue [W^7]*KR12*-KAEK.

Microorganism	Strain	MIC values KR12 Peptide (μg ml^{-1})	MBC values KR12 Peptide (μg ml^{-1})	MIC values [W^7]*KR12*-KAEK (μg ml^{-1})	MBC values [W^7]*KR12*-KAEK (μg ml^{-1})
Streptococcus mutans	ATCC 25175	-	-	31.25	62.5
Streptococcus mutans	UA 159	-	-	31.25	31.25
Streptococcus mutans	UA 130	-	-	7.8	15.6

Antibiofilm activity

Analysis of quantification of biomass

Referring to KR12 peptide, as already expected due to the results of MIC and MBC tests, were not visualized significant interference in biofilm formation of *S. mutans* (Figure 4A-4C), except for the strain UA130 which showed a reduction of approximately 20% in the formation of biomass when subjected to native peptide at a concentration of 250 μg ml^{-1}, compared to the negative control (Figure 4A).

However, the peptide [W^7]*KR12*-KAEK showed effective inhibition of the growth of biofilms for 24 hours. Analyses of the data show a significant difference ($p<0.001$) compared to the negative control (Figure 4D-4F).

The peptide [W^7]*KR12*-KAEK demonstrated the potential to inhibit biofilm formation in all bacterial strains tested. The strains *S. mutans* UA130 and UA159 were lightly more susceptible to [W^7]*KR12*-KAEK*;* as all concentrations tested were able to significantly reduce microbial biofilm. Furthermore, at concentrations above 31.25 μg ml^{-1} there was a reduction of the biofilm by 96-100% (Figure 4D and 4E).

Finally, *S. mutans* ATCC25175 showed a slightly different pattern of susceptibility from the other two strains analyzed. At concentrations ranging from 15.6 to 31.25 μg ml^{-1}, [W^7]*KR12*-KAEK interfered significantly in biofilm formation, with biomass reductions ranging from 48-74%. Concentrations above 62.5 μg ml^{-1} were more effective, causing biomass reductions of 99-100%, with no significant difference from the positive control. Nevertheless, at a concentration of 7.8 μg ml^{-1}, the synthetic peptide caused a statistically significant increase in biomass formation when compared to the negative control (Figure 4F).

Counts of Colony Forming Units

Counts of CFU were performed for all concentrations tested for the native peptide KR12 (Figure 5A-5C) and only at concentrations below the MBC for the peptide [W^7]*KR12*-KAEK because, at higher concentrations, there were no viable colonies to count due to its bactericidal activity (Figure 5D-5F).

Similar to previous tests, the KR12 peptide showed no antimicrobial activity against the strains tested, except for *S. mutans* UA130 that, at concentration of 250 μg ml^{-1}, the peptide generated a significant reduction in the count of colony forming units.

The peptide [W^7]*KR12*-KAEK showed a dose-dependent activity against all microbial strains tested. *S. mutans* UA130 showed the greatest susceptibility to the peptide, significantly reducing the number of viable bacterial cells at very low concentrations, 15.6 μg ml^{-1} despite the discrete biomass formation found in the previous test (Fig. 5D). For *S. mutans* UA159 the results were consistent with the reduction of biomass found previously; with a reduction in viable cells of 82% at the concentration of 15.6 μg ml^{-1} and 31% for the concentration of 7.8 μg ml^{-1} (Fig. 5E).

Although *S. mutans* ATCC25175 demonstrated lower susceptibility to [W^7]*KR12*-KAEK, the number of viable cells in the biofilm was reduced. At concentrations of 15.6 and 31.25 μg ml^{-1}, the peptide reduced the number of cells by approximately 41% and 67%, respectively. However, an interesting result was found at concentration of 7.8 μg ml^{-1}. Even with a high quantity of biomass found on the previous test, the viable cells were reduced by approximately 40% (Fig. 5F).

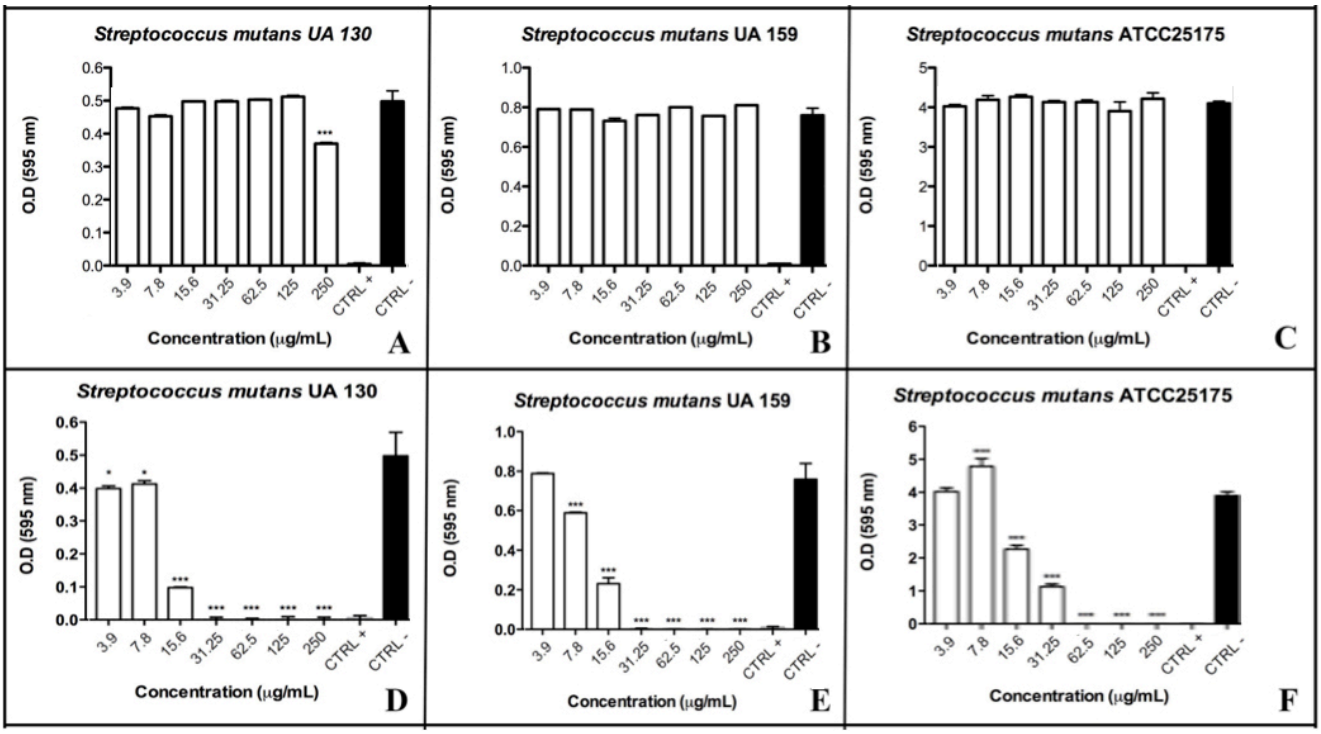

Figure 4: Quantification of biomass for analysis of antibiofilm activity. Effect of KR12 peptide (A-C) and its analogue [W^7]*KR12*-KAEK (D-F) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▣). *p<0.05; ***p<0.001.

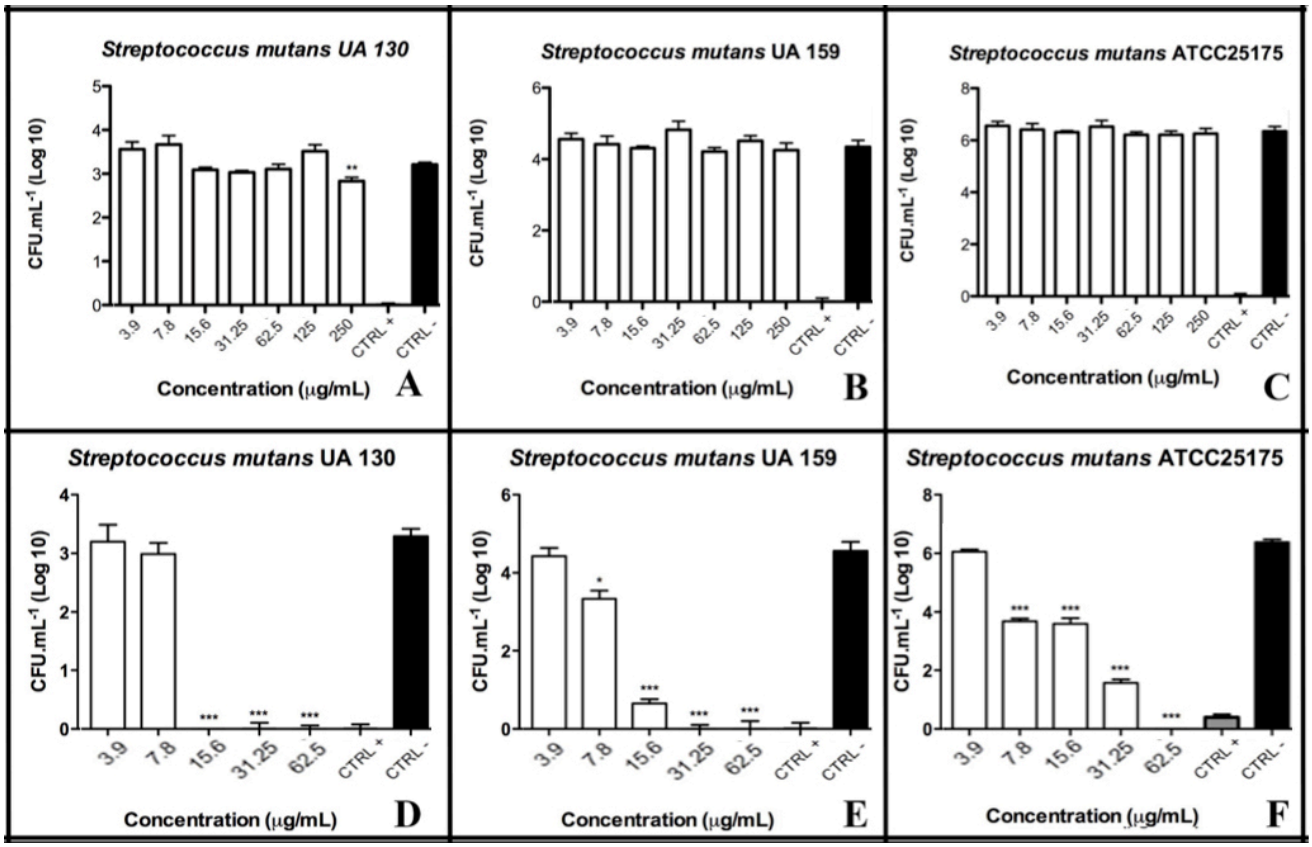

Figure 5: Counting of colony forming units for analysis of antibiofilm activity. Effect of KR12 peptide (A-C) and its analogue [W[7]]*KR12*-KAEK (D-F) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▣). *p<0.05; **p<0.01; ***p<0.001.

Activity of the synthetic peptide on pre-formed biofilm

Tests on pre-formed biofilms were performed for both peptides (Figures 6 and 7). For KR12 peptide, the results were as expected with no significant antimicrobial activity. The strain S. mutans UA130 showed biomass increases in some concentrations, but with no accompanying increase of colony forming units in the same peptide concentrations (Fig. 6A and 6D). For S. mutans UA159, a slight decrease in biomass was noted at the highest peptide concentration, but with no reduction in the number of viable cells either (Fig. 6B and 6E).

The results found regarding the activity of the synthetic peptide on pre-formed biofilm was at least interesting. Regarding the quantification of biomass, any remarkable results were found for all the strains tested (Fig. 7A-7C). However, when performed the viable cell count, it was noticed a remarkable antibacterial activity.

Just as in the previous results, *S. mutans* UA 130 shown a considerable susceptibility to the peptide, with a reduction ranging from 21% at a concentration of 62.5 μg ml^{-1} and 81% at the highest concentration tested; the latter, with a statistically better result than the positive control (Fig. 7D).

For the UA 159 strain, significant activity was noted at concentrations between 125 and 500 μg ml^{-1}, with a considerable reduction in the amount of viable cells. At the concentration of 500 μg ml^{-1}, the result was statistically similar to the positive control (Fig. 7E).

As expected, the most resistant strain against the effect of [W[7]]*KR12*-KAEK was *S. mutans* ATCC25175. Only at concentrations of 500 μg ml^{-1} a statistically significant reduction was found (36%). However, without statistical difference for the positive control (Fig. 7F).

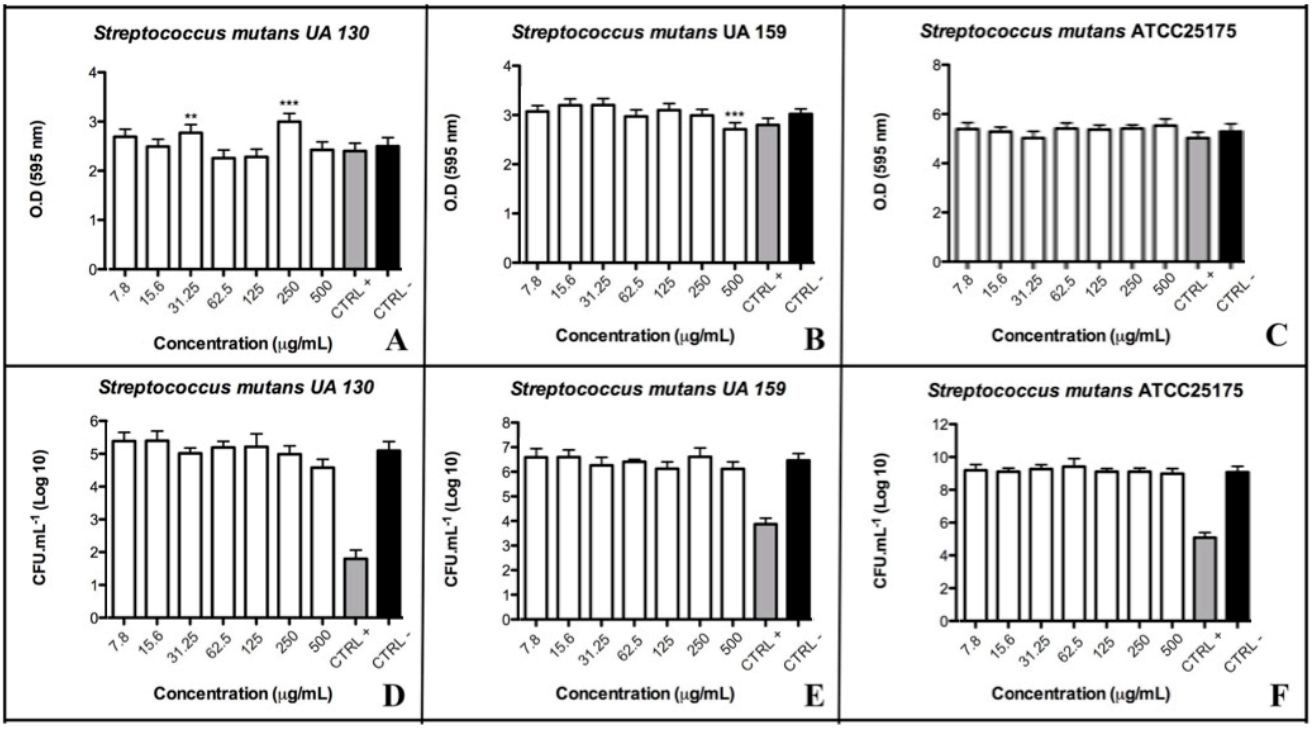

Figure 6: Antibiofilm activity of KR12 peptide on pre-formed biofilms. Quantification of biomass (A-C) and Counting of colony forming units (D-F) of the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▒). **p<0.01; ***p<0.001.

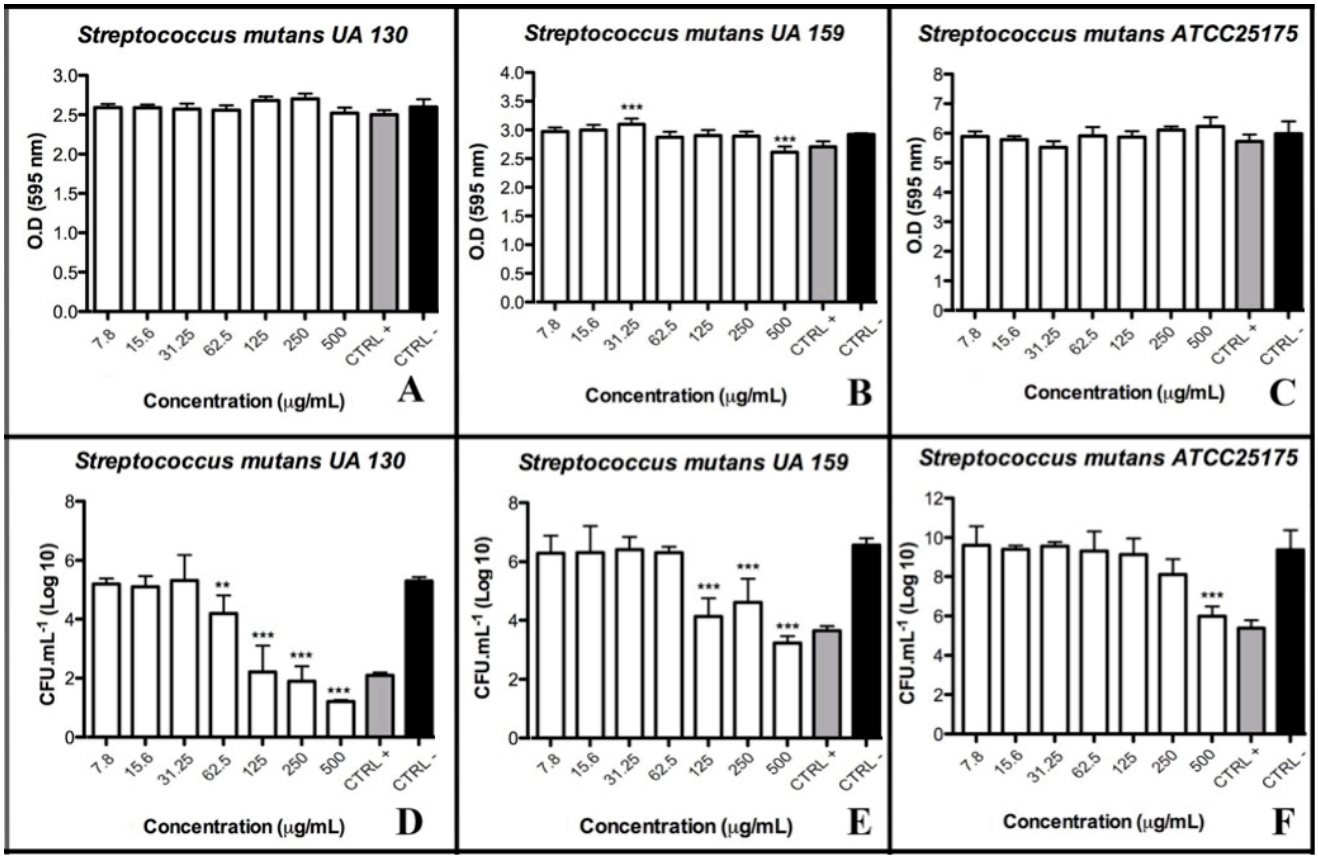

Figure 7: Antibiofilm activity of [W^7]*KR12*-KAEK peptide on pre-formed biofilms. Quantification of biomass (A-C) and Counting of colony forming units (D-F) of the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▒). **p<0.01; ***p<0.001.

Scanning electron microscopy of pre-formed biofilm

The images obtained by scanning electron microscopy were quite interesting. In 8A and 8B figures, it was observed an intense biofilm formation by the strain *S. mutans* UA130 upon incubation with the negative control. It was also observed a high deposition of polymer extracellular matrix, in the increase of 3000x.

During analysis of the pre-formed biofilm from the same strain when incubated with the tested peptide (Fig. 8C and 8D), it could be noted a smaller microcolonies formation associated with a lower deposition of matrix. Furthermore, at the increase of 20000x, some cells were seen with apparent morphological changes suggesting a membrane alteration caused by the peptide.

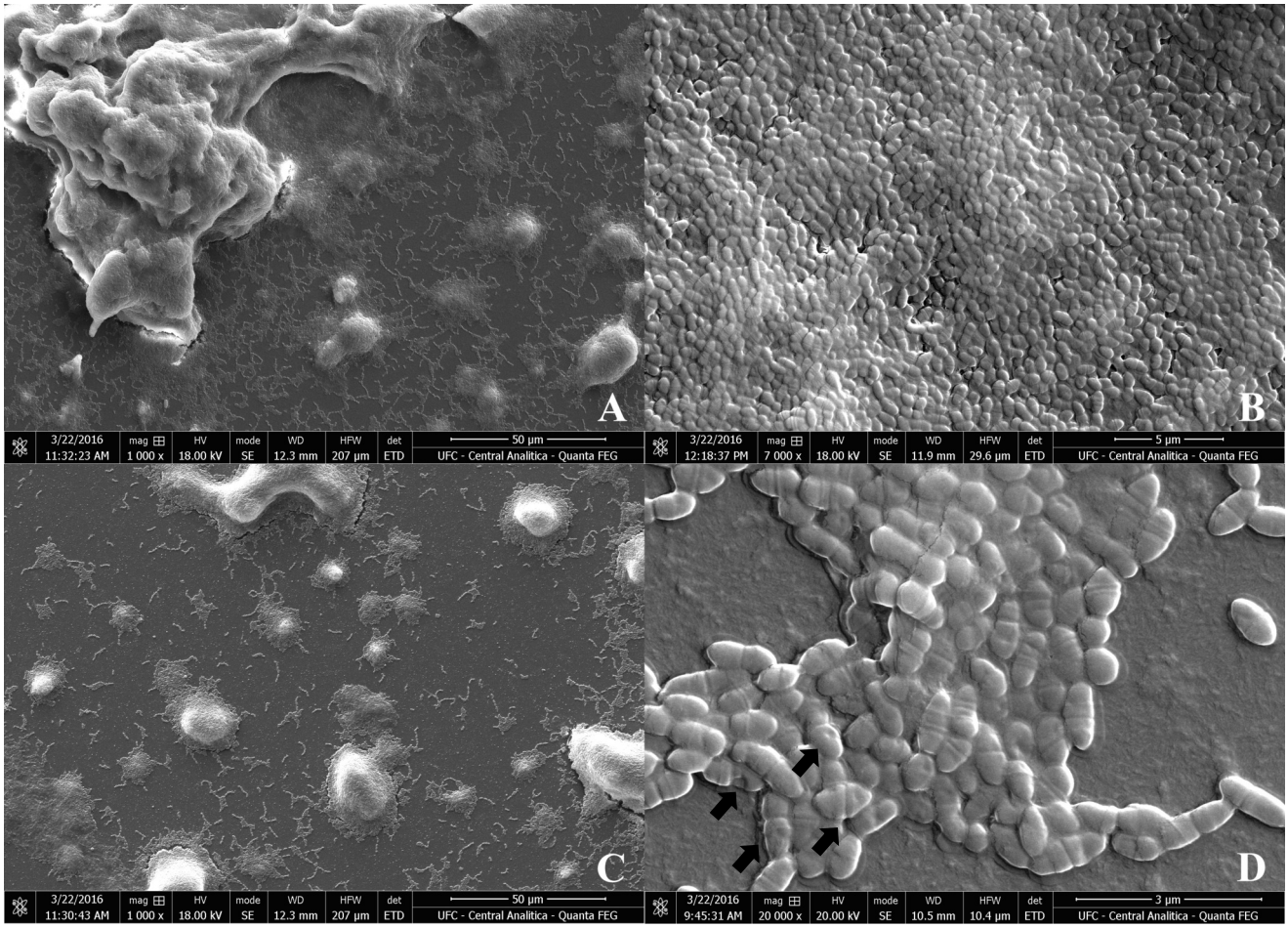

Figure 8: Images by scanning electron microscopy. Pre-formed biofilm of *S. mutans* UA130 incubated with negative control only (A and B). Pre-formed biofilm of *S. mutans* UA130 after incubation with the tested peptide at a concentration of 500 µg ml^{-1} (C and D). Black arrows indicate morphological changes in bacterial structure.

Discussion

The great effectiveness of the natural antimicrobial peptides (AMPs) justifies the research effort to elucidate their function and eventually turning them into new antimicrobial agents against microorganisms resistant to conventional antibiotics (Mishra et al. 2013). The participation and contributions of many laboratories around the world led to a substantial increase in the number of such

peptides. Until June 2016, there were 2260 AMPs in antimicrobial peptide database (APD), however, of these, only 21 have antibiofilm activity scientifically proven to date (*http://aps.unmc.edu/AP/main.php*).

Meanwhile, synthetic modified peptides arise, molecules with changes in its primary structure that allow an increase in its antimicrobial activity and reduces its potential cytotoxicity.

The KR-12 peptide is one of the lowest antimicrobial peptides which are known to date and one of the main factors that make it an interesting model to study is their relative selectivity for Gram-negative bacteria (Wang 2008; Wang et al. 2012a). However, changes in native peptides can effectively change their profile of action and its antimicrobial potential.

Until then, the scientific literature shows that the native peptide KR-12 and its analogs or derivatives, have never been tested against biofilms of oral bacteria. In this study, through the addition of certain amino acids at specific positions, we observed a significant increase in the antimicrobial activity of the peptide, increasing its spectrum of action to bacteria previously not susceptible to it.

Trp is of particular interest due its propensity to position itself near the membrane/water interface. In other studies, the Trp addition has promoted an increase of biological activity. This result was found with Hylin-a1 peptide (Crusca et al. 2010), were the peptide W^6-Hy-a1 was more active than Hy-a1. We also added the sequence KAEK at C-terminus position of KR-12 increasing the chain size of the peptide and its amphipathicity. This modification also increased the charge of peptide. Studies showed that biological activity of antimicrobial peptides generally correlates with the propensity for helical formation, hydrophobicity, cationic property and amphipathicity (Chen et al. 2005; Cespedes et al. 2012). These properties are directly related to the peptide's ability to interact with the bacterial membrane and form pores that lead to cell lysis and death.

Among the main mechanisms suggested for the biocide activity of AMPs, there are the models of extracellular cell death and the models for intracellular cell death. Regarding the models for extracellular cell death, the AMPs are initially attracted to the surface of the microorganism by electrostatic interactions between the anionic or cationic peptide and structures on the cell surface. After the initial attraction, the AMP binds to the cell surface and initiates the connection phase stage that is responsible for performing the crossing of the AMP across the outer membrane and allows the AMP to interact directly with the bacterial cytoplasmic membrane. At low peptide/lipid ratios, the AMPs are connected in parallel to the lipid bilayer. As this ratio increases, the peptides begin to orientate perpendicularly to the membrane. As the peptide/lipid ratios are high, the AMP start its penetration into the cell membrane, which leads to the formation of transmembrane pores, which subsequently leads to cell death by loss of cytoplasmic membrane by breaking liquid and micellization (Brogden 2005; Paulsen et al. 2013). This model of action explains the morphological changes displayed by scanning electron microscopy (Figure 8D).

While lysis of the cell membrane mild to bacterial death, an increasing number of studies have reported the existence of other methods of cell death is remarkable. Researches have revealed the presence of significant intracellular targets for cell death (Futaki et al. 2001; Richard et al. 2003; Wadia et al. 2004). Although we have no evidence of this activity, the results show a reduction in the production of biomass by the evaluated microorganisms which may suggest a possible intervention of the test peptide in this metabolic pathway, as well as other molecules described in the literature (Brötz et al. 1998; Patrzykat et al. 2002).

Streptococcus mutans is one of the most studied micro-organisms in the field of dentistry because of its intrinsic participation in the cariogenic process, although other microorganisms are also related to

the decrease in pH locally on the tooth surface (such as bacteria of the genus *Actinomyces*, *Lactobacillus* and *Bifidobacterium* (van Ruyven et al. 2000).

Because of the role of *S. mutans* in dental demineralization process, several studies propose new control methods to microbial colonization. With respect to antimicrobial peptides, several studies such as Silva et al., (2013); Wang et al. (2012b); Zhang et al. (2016) evaluated the antimicrobial potential of such molecules against strains of S. mutans. However, few of these studies evaluated the antibiofilm effect of AMPs either by interfering in the formation of these communities, or through its effect on pre-formed biofilms.

In a study by Napimoga et al. (2004), microbiological samples were collected from different sites of carious individuals with different risk for the development of dental caries. After analysis of these samples, it was possible to verify that the main strains found were of the species *S, mutans* UA159 and ATCC 25175. However, when the relationship of this species with the caries risk factor it was evidenced a direct relationship of the strain ATCC 25175 with the more aggressive lesions and patients with high caries index. Furthermore, after genotypic comparison of three strains of the same species (ATCC 25175, UA159 and T8), It was noted, in the strains ATCC 25175, a wide range of mechanisms related to microbial resistance not present in the other strains (Napimoga et al. 2004). These findings corroborate the results found in this study regarding to antimicrobial activity only at higher concentrations for *S. mutans* ATCC 25175.

During this study, it was realized that, in many situations under sub-inhibitory concentrations, there was an increase in the quantification of biomass, although it has been found significant decreases in the number of colony forming units in the same concentrations. This was noticed both in inhibitory activity assays of biofilm as in assays for preformed biofilms. This finding suggests that, under stress of the antimicrobial agent, the remaining cells and, consequently, more tolerant ones, increase the production of extracellular polymer matrix as a defense mechanism to the aggressive agent. This finding was also evidenced by Liu and Burne (2009) as well as by Wu et al. (2010).

Conclusion

Based on the results, it could be concluded that the modified peptide [W^7]*KR12*-KAEK It has antimicrobial and antibiofilm activity against the tested bacterial strains, unlike the natural peptide KR-12.

However, despite the visual membrane changes, further studies on the mechanism of action and cytotoxic activity still need to be conducted in order to develop an effective commercial product in the control and/or treatment of cariogenic infections.

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Capítulo IV – Artigo Científico II

The synthetic peptide [W^7]KR12-KAEK has antimicrobial and antibiofilm activity against Enterococcus faecalis strains.

Proposta de Submissão: Archives of Oral Biology (B2 Biotecnologia / Fator de Impacto: 1.733)

Research Article

The synthetic peptide [W^7]*KR12*-KAEK has antimicrobial and antibiofilm activity against *Enterococcus faecalis* strains.

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Abstract

Objective: The purpose of this study was to evaluate the antimicrobial and antibiofilm activity of the native peptide KR-12 and its derivative the synthetic peptide [W^7]*KR12*-KAEK on the planktonic and biofilm growth of *Enterococcus faecalis* strains.

Design: The methods used to evaluate antimicrobial activity include the following: determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in microtiter plates for growth in suspension; and quantification of biomass by crystal violet staining and counting of colony forming units for biofilm growth. In addition, structure assessments of biofilm and cellular morphological changes were analyzed by scanning electron microscopy . The microorganisms grown in Brain Heart Infusion broth at 37 °C under atmospheric pressure with 5% CO_2. The peptides were solubilized in 0.1% acetic acid (v/v) at various concentrations (500 to 3.9 μg/ml). Chlorhexidine gluconate 0.12% was used as the positive control, and BHI culture medium was used as the negative control.

Results: The tested peptide [W^7]*KR12*-KAEK demonstrated a remarkable antimicrobial and antibiofilm effects, inhibiting the planktonic and biofilm growth of all strains tested, even at low concentrations.

Conclusion: The peptide [W^7]*KR12*-KAEK is a potential biotechnological input for the development of auxiliary antimicrobial therapies for endodontic treatment.

Keywords: Antimicrobial Peptide; *Enterococcus faecalis*; Biofilm

1. Introduction

Endodontic treatment is one of the most common procedures in dentistry nowadays. In recent study, it was found, through a systematic review of the literature, that 5% of all teeth evaluated by the researchers had some type of periapical radiolucency and 10% of all teeth already had endodontic treatment concluded (Pak, Fayazi, & White, 2012).

In general, the need for endodontic treatment is derived from an infectious process. Thus, the main objective of this treatment is the complete disinfection of the whole root canal, however, due to the complex root anatomy, many times such disinfection is a challenge to the dentistry professional (Venkateshbabu et al., 2016).

The root canal can be considered an extremely harsh environment due to their strict conditions of oxygenation and nutrition, in addition to presenting pH variations depending on the root portion involved (Sundqvist, 1992). Thus, researchers believe that only certain microorganisms with increased virulence and adaptive capacity are capable of colonizing the root canal and forming biofilms in its interior (Kassen & Rainey, 2004; Raskin, Seshadri, Pukatzki, & Mekalanos, 2006).

In this perspective, *Enterococcus faecalis* emerges as one of the main pathogens associated with endodontic infections and one of the most studied in the field. Several studies show that *E. faecalis* is present in 32-82% of endodontic lesions (Molander, Reit, Dahlen, & Kvist, 1998; Peciuliene, Balciuniene, Eriksen, & Haapasalo, 2000; Gomes et al., 2006) and, due to its antibacterial properties, it

is one of the main microorganisms related to persistent endodontic lesions or the need for endodontic retreatment (Rôças, Siqueira, & Santos, 2004; Stuart, Schwartz, Beeson, & Owatz, 2006).

Due to the infectious nature of the disease and the presence of biofilms in root canals, endodontic treatment aims the decontamination by physical methods, but also counts with the assistance of chemical methods that act in canaliculi that conventional instruments do not reach and also because the substantivity of the antimicrobial material within the conduits, keeping their biocide activity for much longer (Darcey et al., 2016; Plotino et al., 2016). Studies show increasingly the importance of finding new effective irrigating solutions for root canal decontamination (Dunavant et al., 2006; Giardino et al., 2007; Garlapati et al., 2016; Gonçalves et al., 2016).

Among the molecules most widely studied recently, we highlight the antimicrobial peptides (AMPs). The AMPs are molecules present in many living organisms produced mainly by the innate immune system of the host against infections (Gorr & Abdolhosseini, 2011). Currently, it is known that these biomolecules may act in different ways, both interacting on bacterial cell membrane as well as affecting intracellular metabolic pathways of these cells. This feature provides a broad spectrum of activity to AMPs (Zasloff, 2002; de Freitas Lima et al., 2015). The low concentration of AMPs to exert antimicrobial activity and tissue repair highlight its potential as a root canal irrigating solutions (da Silva et al., 2012; Winfred et al., 2014).

The antimicrobial peptide KR-12 is considered the lowest natural active peptide known, corresponding to amino acids 18-29 of the human cathelicidin peptide LL-37 (Wang, 2008). Due to its small size, low toxicity and high solubility in culture media, this peptide has served as template in the design of new synthetic antimicrobial molecules (Mishra, Epand, Epand, & Wang, 2013). In research conducted in 2013, it was evident that several analogues of KR-12 peptide were able to exert significant antimicrobial activity without toxicity to mammalian cells (Jacob, Park, Bang, & Shin, 2013).

Thus, the aim of this study was to evaluate the antimicrobial and antibiofilm activity of a synthetic antimicrobial peptide [W^7]*KR12*-KAEK (KRIVQRWKDFLRKAEK-NH_2), based on the primary structure of the native peptide KR-12 on two strains of *Enterococcus faecalis.*

2. Materials & Methods

2.1. Bacterial strains and culture conditions

The *Enterococcus faecalis* strains ATCC 19433 and ATCC 10100 were grown in Brain Heart Infusion Agar (BHIA, Difco, Himedia, India) for 24 h in an atmosphere of 37°C and 5% CO_2. After growth on Agar medium, isolated colonies were removed and inoculated into 5 ml of sterile medium BHI broth under the same conditions. A bacterial cell concentration of 10^6 CFU/ml was established in BHI broth prior to all experiments.

2.2. Peptide synthesis, purification and preparation

The peptide [W^7]*KR12*-KAEK (KRIVQRWKDFLRKAEK-NH_2) were synthesized according to previously published study of Silva et al., 2016 using a Protein PS-3 synthesizer by solid phase peptide

synthesis (Merrifield, 1963) and using standard 9-fluorenylmethyloxycarbonyl (Fmoc) protocols on Fmoc-Arg(Pbf).

The identity of the peptide was confirmed by mass spectrometry in positive-ion mode ESI on a Bruker model apparatus.

The peptide was fully solubilized in sterile deionized water with 0.1% acetic acid (CH_3COOH) at a concentration of 500 μg/ml prior to use and stored in a freezer at -20ºC.

2.3. Antimicrobial activity assay

The microdilution test in 96-well "U" bottom microtiter plates was used for establish the antimicrobial activity of [W^7]*KR12*-KAEK peptide. The first column of each plate were prepared with sterile BHI containing the peptide [W^7]*KR12*-KAEK at a concentration of 500 μg/ml. Then, two-fold serial dilutions were made to achieve varying concentrations (7.8 to 500 μg/ml) at a final volume of 100 μl. Next, 100 μl of cells (2 x 10^6 CFU/ml) was added to each well to yield a final volume of 200 μl. Chlorhexidine gluconate (0.12%) and BHI sterile culture medium were added to separate wells as the positive and negative controls, respectively.

The plates were incubated at 37°C with 5% CO_2 for 24 hours. After incubation period, the bacterial growth was assessed by turbidity of each well, which was measured using a spectrophotometer (SpectraMax® I3) at 620 nm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of inhibiting bacterial growth visually.

To determine the minimum bactericidal concentration (MBC), Petri dishes with BHI agar were inoculated with 10 μl from the wells that showed no visible microbial growth. The MBC was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of completely inhibiting microbial growth on the Petri dishes.

2.4. Antibiofilm activity assay

As for the biofilm formation assays, they were performed according to the methodology described by previous research, in 96-well "flat" bottom microtiter plates with modifications (Stepanovic et al., 2000). Each plate was prepared in the same manner as in the antimicrobial activity test. However, two plates were prepared for each test, one for the quantification of biomass (crystal violet) and one for counting the CFU (Colony Forming Units) adhered to the plate.

2.4.1. Quantification of biomass

After 24 hours of incubation at 37°C, the plates were washed three times with sterile distilled water, and then 200 μl of methyl alcohol P.A. was added to fix adhered cells. After the removal of methanol, 200 μl of 1% crystal violet was added for 5 minutes. This procedure was performed to allow an indirect quantification of biofilm formation by staining with crystal violet. Then, the washing procedure was repeated and the plate placed at room temperature for drying. To dissolve the crystal violet bound to the biofilm, 200 μl of 33% acetic acid were added to wells and the absorbance of each well was measured with a spectrophotometer (SpectraMax® I3) at 595 nm.

2.4.2. Count of Colony Forming Units (CFU)

After incubation for 24 hours at 37°C, the plates were washed three times with sterile distilled water to remove weakly attached cells. Next, 200 µl of sterile 0.9% NaCl solution was added to each well of the plate, and the plate was then subjected to an ultrasonic bath (Sonicor/SC-52) for 6 minutes to release the cells from the formed biofilm. The volume of five wells was removed with an up-down movement and collected into a sterile 2.0 ml microtube to a final volume of 1 ml. Ten-fold serial dilutions of the cell suspension were performed for subsequent plating. Then, the plates were incubated at 37°C with 5% CO_2 for 24 hours. The number of colonies grown on each plate were counted and converted to units of CFU/ml (Log_{10}).

2.4.3. Pre-formed biofilm assay

To evaluate the activity of [W^7]*KR12*-KAEK on bacterial mature biofilms, 200 µl of cells suspension (10^6 cells/ml) were added to each well and incubated for 24 h at 37°C with 5% CO_2. After biofilm development the wells were washed twice with 200 µl of sterile distilled water for removal of non-adhered cells and an aliquot of 200 µl of the peptide solubilized in BHIs (at concentrations ranging from 7.8 to 500 µg/ml) was added to the wells. Chlorhexidine gluconate (0.12%) and BHI sterile culture medium were added to separate wells as the positive and negative controls, respectively. The plates were incubated for 24 h at 37°C in 5% CO_2. After 24 h, the medium was removed and each well was the washed twice with 200 µl of sterile saline solution. The effect of [W^7]*KR12*-KAEK on pre-formed biofilms was evaluated by biomass quantification and count of colony forming units as described above.

2.5. Antibiofilm analysis of [W^7]KR12-KAEK in scanning electron microscopy

The polystyrene plates were set up and incubated as described above for tests in preformed biofilms but testing only de peptide [W^7]*KR12*-KAEK at a concentration of 500 µg/ml and the negative control against the strain *E. faecalis* ATCC 19433.

The plates were removed from incubation and weakly adherent cells were removed by washing with sterile ultrapure water, and then allowed to dry at ambient temperature. Wells containing biofilms were dehydrated sequentially with ethanol (70% ethanol for 10 minutes, 95% for 10 minutes and 100% for 20 minutes) and allowed to dry at ambient temperature. After drying, they were kept in desiccator until analysis. Before observation, cells were mounted on aluminum holders and covered with gold particles and then observed under the scanning electron microscope S-360 (Leo, Cambridge, USA).

2.6. Statistical analysis

All experiments were performed in triplicate, and the results were entered into Microsoft Excel (Version 2010 for Windows) and later analyzed with GraphPad Prism software (version 5.0 for Windows). To analyze the significant differences between groups, one-way ANOVA was conducted with a Bonferroni post-test, and $p<0.001$ was considered significant.

3. Results

3.1. Antimicrobial activity

Regarding the activity on microorganisms in planktonic state, the peptides KR-12 and [W^7]*KR12*-KAEK showed considerably different activities among themselves on the tested bacterial strains.

While the KR-12 peptide did not exhibit any bactericidal or bacteriostatic activity against the microorganisms tested at the concentrations used, the modified peptide was capable of inhibiting bacterial growth from the concentrations of 15.6 µg/ml and 31.25 µg/ml for *E. faecalis* ATCC 19433 and ATCC 10100, respectively. However, at the concentration of 62.5 µg/ml, the peptide exerted bactericidal activity for both strains analyzed (Table 1)

Table 1: *In vitro* susceptibility of oral microorganisms tested against the peptides KR-12 and [W^7]*KR12*-KAEK.

Microorganism	Strain	MIC values KR-12 Peptide (µg/ml)	MBC values KR-12 Peptide (µg/ml)	MIC values [W^7]*KR12*-KAEK (µg/ml)	MBC values [W^7]*KR12*-KAEK (µg/ml)
Enterococcus faecalis	ATCC 10100	-	-	31.25	62.5
Enterococcus faecalis	ATCC 19433	-	-	15.6	62.5

3.3. Antibiofilm activity

3.3.1. Analysis of quantification of biomass

For biofilms inhibition tests, due to the results previously shown, it was expected that the native peptide did not exhibit any kind of interference in this process (Figures 1A and 1B).

However, when we observe the results about the peptide [W^7]*KR12*-KAEK, it is evident that it exhibited significant activity in biomass reduction in both strains tested.

For the strain *E. faecalis* ATCC 10100, there was an interference of 98% in the production of biomass after 24 hours of growth in contact with the synthetic peptide. This interference was observed in concentrations above 62.5 µg/ml. Such result was not statistically different when compared to the positive control. One of the interesting findings of this analysis was that in lower concentrations there were not any kind of interference in the biomass itself, although the concentration of 31.25 µg/ml is capable of inhibiting bacterial proliferation in planktonic state as previously seen.

Regarding the results obtained about the strain *Enterococcus faecalis* ATCC 19433, there was a dose-dependent activity of the peptide [W^7]*KR12*-KAEK. The concentrations between 31.25 – 250 µg/ml were able to completely inhibit biofilm formation without statistical differences of the positive control. However, at lower concentrations of 15.6 and 7.8 µg/ml, there was a decrease in the quantification of biomass of 77% and 41%, respectively. In this specific case, the concentration of 15.6 µg/ml, for being the minimum inhibitory concentration, displayed an expected result in significantly interfering biomass production, while at the concentration of 7.8 µg/ml, an subinhibitory concentration, The tested peptide was also able to disturb the formation of bacterial biofilms.

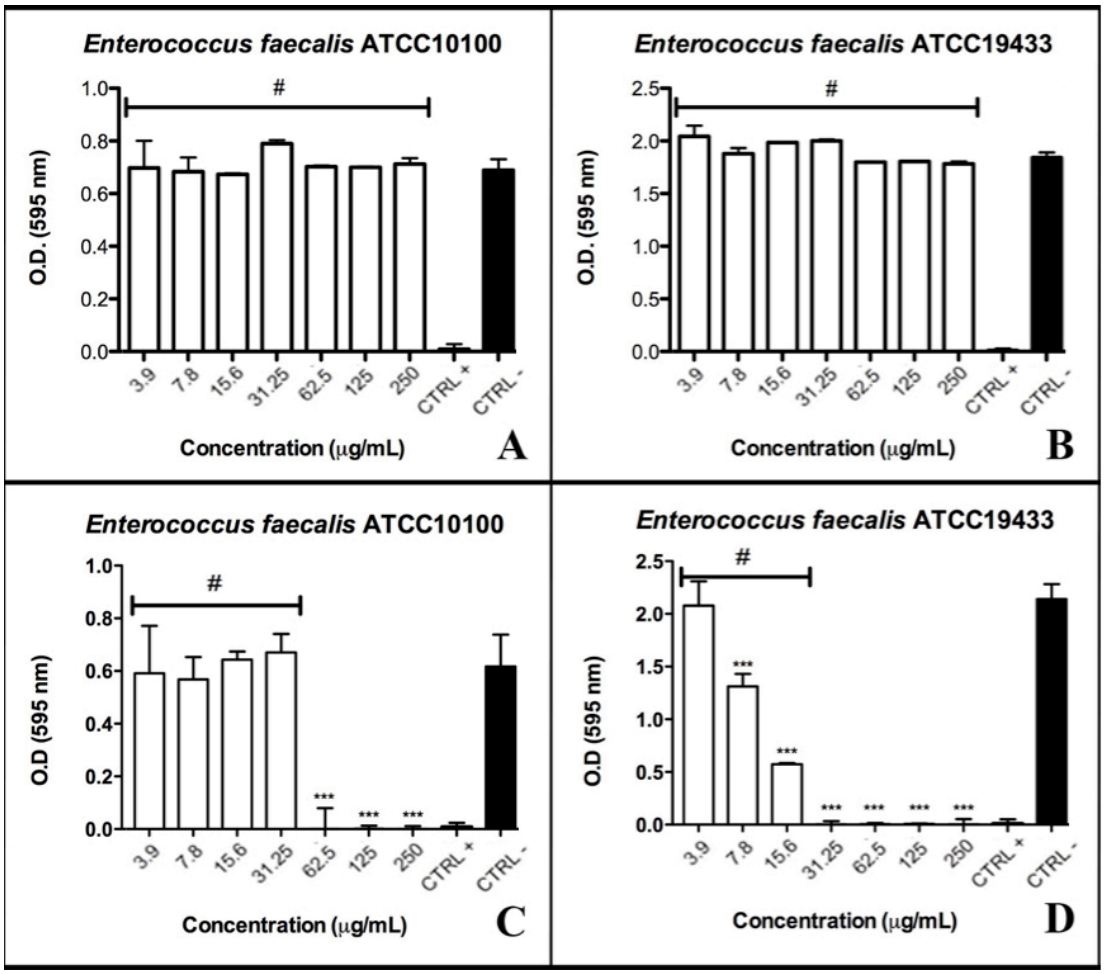

Figure 1: Quantification of biomass for analysis of antibiofilm activity. Effect of KR12 peptide (A-B) and its analogue [W[7]]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▣). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

3.3.2. Counts of Colony Forming Units

The testing for colony forming unit count serves to cell death analysis within the biofilm structure itself. Thus, based on results of quantification of biomass, the couting of CFU were performed for all concentrations tested for the native peptide KR12 and only at concentrations below the MBC for the peptide [W[7]]*KR12*-KAEK because, at higher concentrations, there were no viable colonies to count due to its bactericidal activity (Figure 2).

Corroborating with the results obtained in the tests for quantification of biomass, the peptide native KR-12 exerted no antimicrobial activity in bacterial cells embedded in biofilm (Figures 2A and 2B). However, when we analyze the results of the [W[7]]*KR12*-KAEK peptide, we perceive a bactericidal activity in concentrations higher than 62.5 μg/ml for both strains tested. Furthermore, specifically for the strain ATCC 10100, despite the biomass quantification analysis shows no reduction at the concentration of 31.25 μg/ml, a significant reduction in the number of living cells is noticeable within the biofilm itself (Figure 2C). As for the strain *E. faecalis* ATCC 19433, at the same concentration, the result was the opposite. Although in the biomass analysis, there had been detected no biofilm formation, few living cells still were present at the well surface. This finding demonstrates that, most

likely, although some cells remain viable and could still adhere to the surface, they were unable to form a mature biofilm itself.

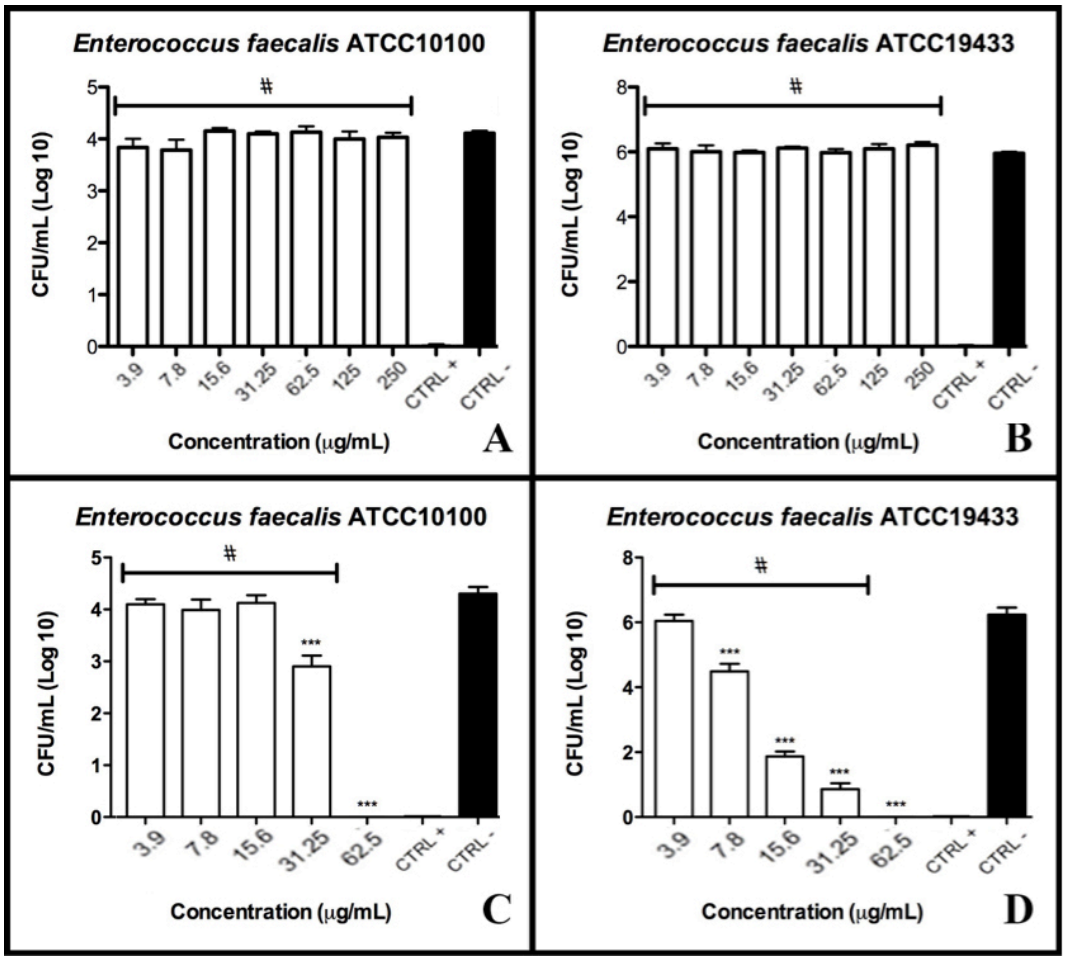

Figure 2: Counting of colony forming units for analysis of antibiofilm activity. Effect of KR12 peptide (A-B) and its analogue [W^7]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▣). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

3.3.3. Activity of the synthetic peptide on pre-formed biofilm

To evaluate the antibiofilm power of the native and synthetic peptides, biomass quantification assays and counting of colony forming units were also conducted on pre-formed biofilms.

Referring to biomassquantification assay, 24hs after application of KR-12 peptide, it was observed no noticeable reduction in the biomass produced. However, a curious fact is highlighted in this assay. The positive control used significantly stimulated the production of biomass from both bacterial strains tested. In a way that, even without biomass reduction at the tested peptide concentrations, compared to negative control, the results were statistically better than the obtained with the positive control (Figures 3A and 3B).

Similar results were found for the peptide [W^7]*KR12*-KAEK. In any of the tested concentrations a statistically significant reduction was observed, when compared with the negative control. However, due to the increased biomass generated by Chlorhexidine gluconate 0.12%, all concentrations tested were statistically better than the positive control.

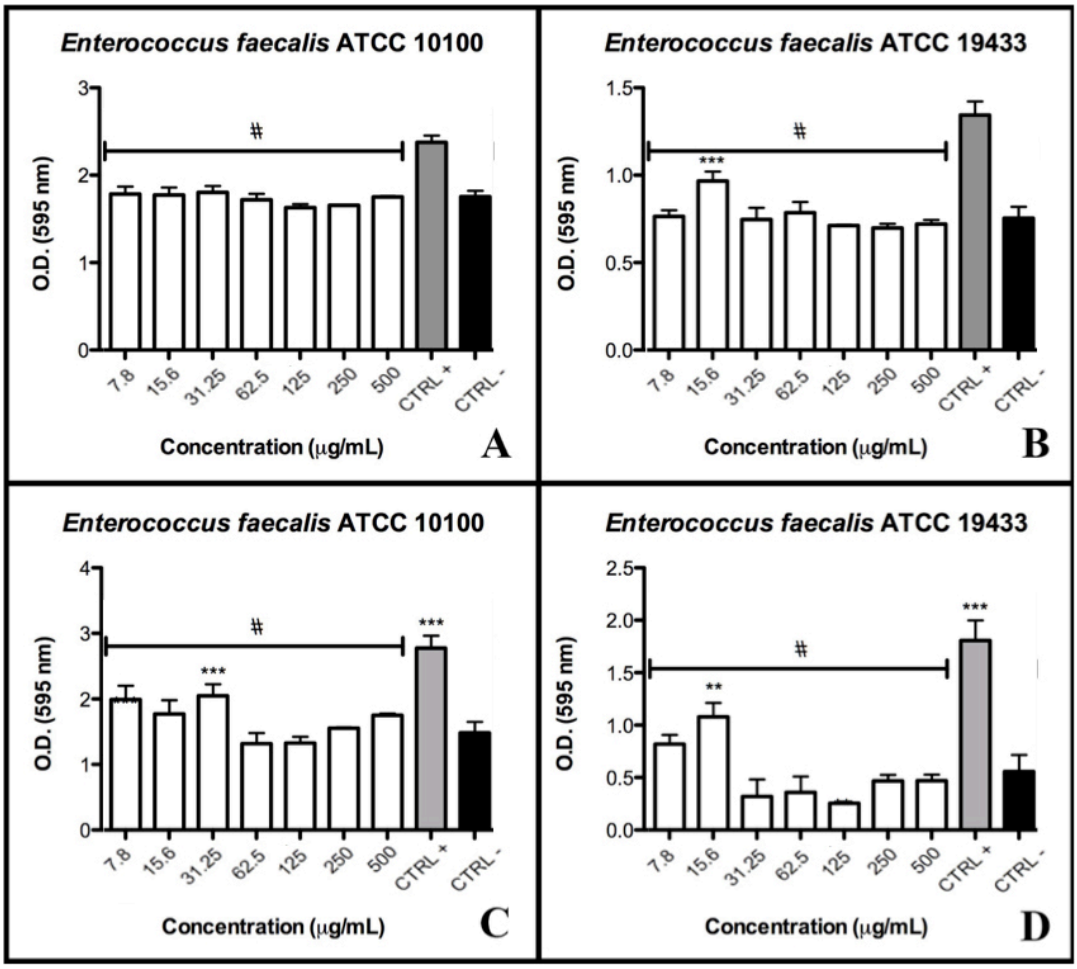

Figure 3: Quantification of biomass for analysis of antibiofilm activity in pre-formed biofilms. Effect of KR12 peptide (A-B) and its analogue [W[7]]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▭). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

Regarding the findings in the couting of colony forming units, they were at least interesting. Despite the significant increase in biomass caused by the positive control, the amount of living cells within the biofilm is considerably low. This demonstrates the antimicrobial power of Chlorhexidine Gluconate.

As for the KR-12 peptide, no reduction in the number of bacterial cells was noteworthy (Figures 4A and 4B), except for the concentration of 500 μg/ml over *E. faecalis* ATCC 19433, in which a slight decrease was observed (Figure 4B).

The most intriguing results were of the peptide [W[7]]*KR12*-KAEK. Although no changes have been detected in biomass quantification tests, when we observed the results of colony forming units count, it is clear that in certain concentrations, there were considerable reductions. For *E. faecalis* ATCC 10100, the higher concentration tested (500 μg/ml) was capable to reduce by approximately 70% the number of live bacterial cells embedded in the mature biofilms. Moreover, at concentrations between 62.5 and 250 μg/ml an average reduction of 19% was obtained. However, regarding the strain *E. faecalis* ATCC 19433, its susceptibility to the tested peptide was considerably higher. Almost all concentrations tested (15.6 – 500 μg/ml) showed a significant decrease in bacterial cell count within the biofilm. Of note was the highest concentration, 500 μg/ml, which was statistically similar to the positive control used.

3.3.4. Scanning electron microscopy of pre-formed biofilm

In Figure 5, it can be observed the structures from mature biofilms of *E. faecalis* ATCC 19433 under both growth: only with the negative control (A and B) as well as with the synthetic peptide [W^7]*KR12*-KAEK (C and D).

In the images A and B, there is, under different magnifications, an intense cell proliferation along with a high production of polymeric extracellular matrix. While in C and D images, it is observed that, after the contact time with the tested peptide, the general structure and amount of the biofilm formed are not affected, however, a decrease in extracellular matrix is visible at a magnification of 20000x as well as changes in the morphology of the bacterial cell membrane, indicating possible peptide interaction with the cellular membrane.

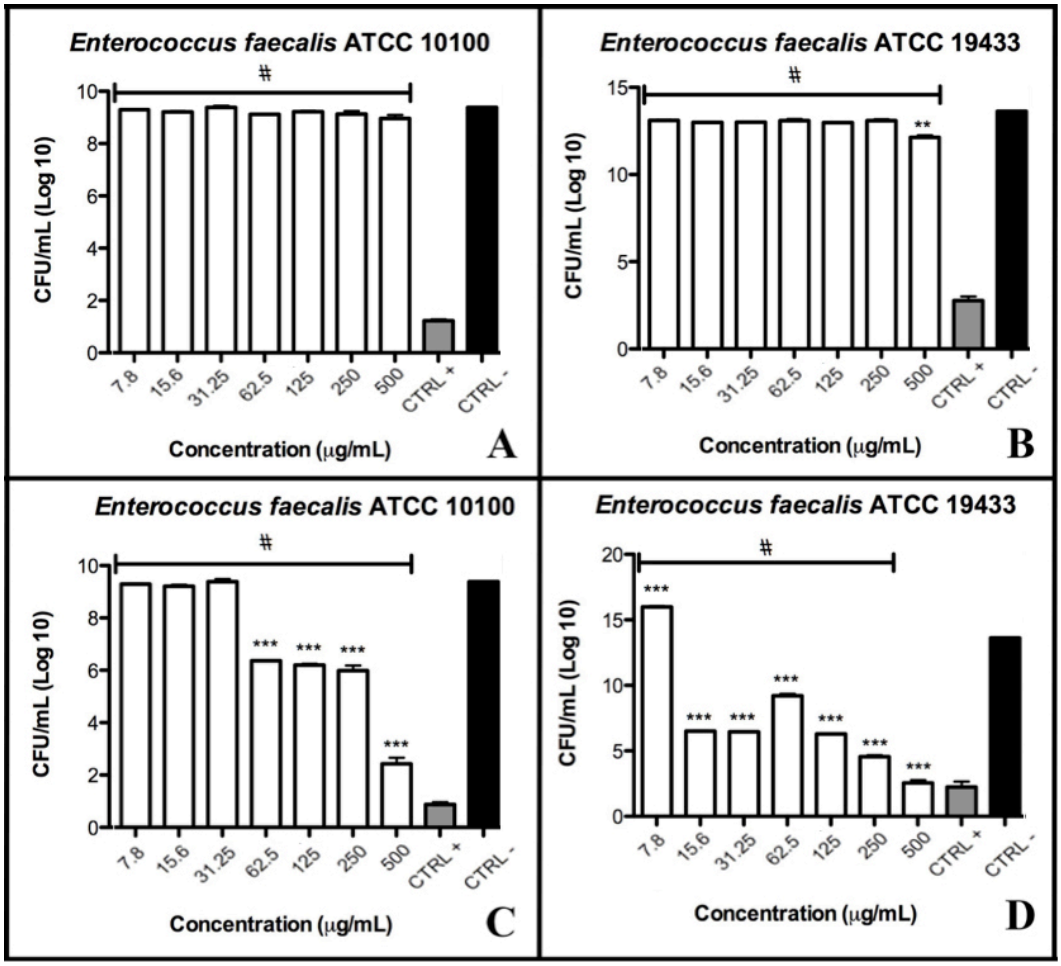

Figure 4: Counting of colony forming units for analysis of antibiofilm activity in pre-formed biofilms. Effect of KR12 peptide (A-B) and its analogue [W^7]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▢). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

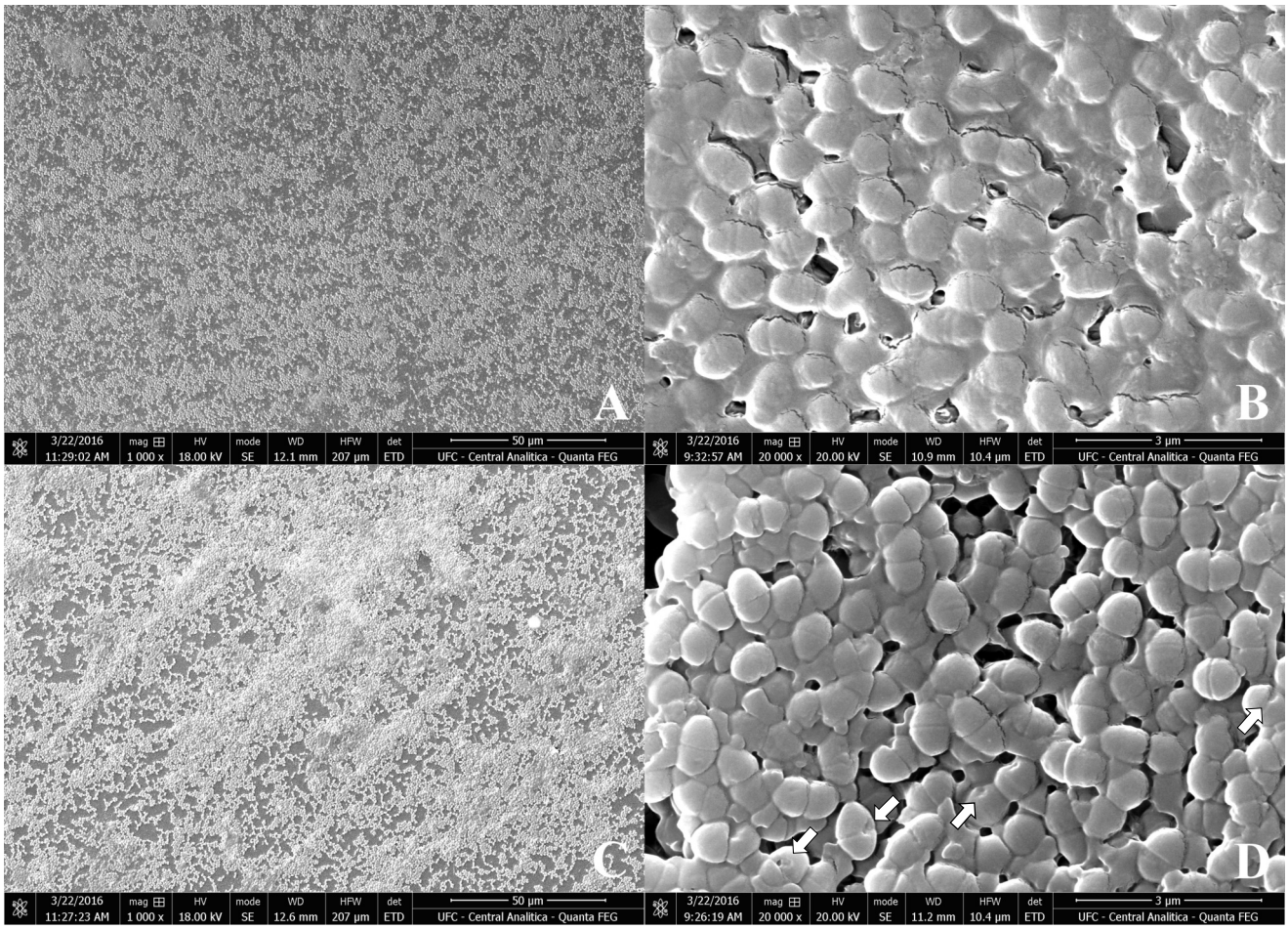

Figure 5: Images by scanning electron microscopy. Pre-formed biofilm of *Enterococcus faecalis* ATCC 19433 incubated with negative control only (A and B). Pre-formed biofilm of *Enterococcus faecalis* ATCC 19433 after incubation with the tested peptide at a concentration of 500 µg/ml (C and D). White arrows indicate morphological changes in bacterial structure.

4. Discussion

Carious lesions are the main cause of pulpal infection and its progression is directly dependent on the virulence and proliferation rate of these microorganisms. These microorganisms then begin a pulpitis process characterized by inflammation and intense neurogenic pain, which culminate in need of endodontic treatment (Zero, Zandona, Vail, & Spolnik, 2011).

Endodontic treatment aims to achieve complete repair and healing of periapical tissues. This process generally follows a sequence of procedures such as instrumentation, irrigation/aspiration, intracanal dressing and filling of the root canal system (de Freitas Lima et al., 2015). A major difficulty of this therapy is the elimination of resident bacteria in the ramifications of the main root canal and in the dentinal tubules. Thus, the intracanal dressing with antimicrobial medications and the irrigating solutions are fundamental in antimicrobial therapy in the endodontic treatment (Darcey et al., 2015).

The search for more biocompatible molecules that have ideal antimicrobial characteristics is on the rise. Several studies researching new natural products such as irrigation or long term intracanal medications can be found (Pujar & Makandar, 2011; Venkateshbabu et al., 2016). However, within the scope of new natural molecules to antimicrobial treatment, antimicrobial peptides emerge as a potential biotechnological agent (da Silva et al., 2012; de Freitas Lima et al., 2015).

In recent study, it was evaluated the antimicrobial potential of LL-37 peptide on two strains of *Enterococcus faecalis* (FA2-2 and OG1X). In that study, it was found that in concentrations greater than 12.5 and 50 μg/ml, the tested peptide exerted antimicrobial activity on the two bacterial strains, respectively (Thennarasu et al., 2010).

In other research conducted by Korean authors, human defensins 1, 2, 3 and 4 were tested on the *E. faecalis* ATCC 29212 strain. In this case, all peptides possessed some antimicrobial activity, being capable of interfering in the bacterial growth at concentrations superior than 2.5 μM (Lee & Baek, 2012).

However, none of these studies evaluated the antibiofilm activity of antimicrobial peptides on strains of E. faecalis, one of the main pathogens of persistent endodontic lesions. In this research were evaluated not only the antimicrobial potential of KR-12 native peptide and its synthetic derivative [W^7]*KR12*-KAEK, as well as their antibiofilm activities against two strains of *E. faecalis*.

One of the major highlights of the present study was the improved antimicrobial activity displayed by the synthetic peptide [W^7]*KR12*-KAEK compared to its native corresponding, KR-12.

Such activity is probably due to addition of certain amino acids at specific locations along the sequence. One, Tryptophan, proves to be important for the peptide interaction with the membrane surface. In a previous study, it was found the prevalence of certain amino acids in many antimicrobial peptides. Of all the amino acids, Tryptophan was the most frequent, and even the most associated with high antimicrobial activity of the peptides. These findings are probably due to the hydrophobic character of this amino acid, influencing the relationship between hydrophobicity and charge of the antimicrobial peptide (Mikut et al., 2015).

In addition to this modification, addition of positively charged amino acids at the N-terminal region of the peptide may also have influenced the increase of antimicrobial activity. It is known that the positive charge on cationic peptides are able to directly change its biocide effect (Silva et al., 2013; Ramesh et al., 2016).

The increased activity of the peptide [W^7]*KR12*-KAEK became clear in the assays against planktonic cells (Table 1) and inhibition of biofilms (Figures 1 and 2). In this latter test, an interesting finding deserves to be highlighted. In Figure 1C and 1D it is clear that even under bacteriostatic concentration, both strains are able to develop biofilm, most notably the strain ATCC10100. However, when we compare the number of viable bacterial cells, there is a reduction in the number of these cells in the concentration of 31.25 μg/ml for *E. faecalis* ATCC10100 and at the concentration of 15.6 μg/ml for *E. faecalis* ATCC19433. This finding was similar to that found in recent research and can be justified by the possible activity time of the tested peptide (Barber, Werth, McRoberts, & Rybak, 2014). If the time for the peptide to develop bacteriostatic activity was greater than the time for cell proliferation, bacterial cells could be multiplied and developed biofilms. This explanation corroborates those results, which show that in bactericidal concentrations, no biomass or viable cell were detected in the tests.

For the tests on preformed biofilms, it was realized that, when in contact with the positive control (Chlorhexidine Gluconate 0.12%) there was an over-production of biomass by the bacteria tested. Such result is consistent with the characteristics of the bacteria *Enterococcus faecalis*. Several studies emphasize its high biomass production capacity in stressful situations (Moura et al., 2015; Pourhajibagher et al., 2016; Strateva et al., 2016).

Such capacity for resistance of E. faecalis to adverse situations can also be seen in Figures 3 and 4. Despite the obvious antimicrobial activity on bacteria embedded in biofilms preformed at concentrations above 62.5 μg/ml for *E. faecalis* ATCC10100 and 15.6 μg/ml for *E. faecalis*

ATCC19433, it is noted that the amount of biomass in this respective concentrations are not significantly different from the negative control. The different susceptibility displayed to the bactericidal activity of the peptide may be due the different gene repertoires between the two analyzed strains (Fisher & Phillips, 2009; Upadhyaya, Lingadevaru, & Lingegowda, 2010).

When observed the cellular changes caused by peptide [W^7]*KR12*-KAEK by scanning electron microscopy, it was noticed a clear reduction in the production of polymeric extracellular matrix, as well as membrane morphological changes. These results are consistent with the possible mechanisms of action of antimicrobial peptides. One of these suggested mechanisms is through direct interaction of the peptide with the bacterial cell membrane and its gradual internalization forming pores that allow the leakage of cytoplasmatic fluid and bacterial structural change (Brogden, 2005; Paulsen et al. 2013). In addition, studies show the activity of certain antimicrobial peptides in bacterial metabolic pathways such as gene expression and protein synthesis (Futaki et al. 2001; Richard et al. 2003; Wadia, Stan & Dowdy 2004).

5. Conclusion

It was concluded, based on the presented results, that the synthetic peptide [W^7]*KR12*-KAEK has antimicrobial and antibiofilm on *Enterococcus faecalis* ATCC10100 and ATCC19433 strains, which highlights it as biotechnological input for production of antimicrobial solutions to aid the endodontic treatment .

However, more studies are needed in order to establish its cytotoxicity and viability in intracanal environment.

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Capítulo V – Artigo Científico III

Antimicrobial and antibiofilm activity of the synthetic peptide [W^7]KR12-KAEK on early colonizers of the oral biofilm.

Proposta de Submissão: Peptides (Qualis B1 Biotecnologia / Fator de Impacto: 2.535)

Research Article

Antimicrobial and antibiofilm activity of the synthetic peptide [W^7]KR12-KAEK on early colonizers of the oral biofilm.

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Abstract

The main objective of this study was to evaluate the antimicrobial and antibiofilm activity of the native peptide KR-12 and its derivative synthetic peptide [W^7]*KR12*-KAEK on the planktonic and biofilm growth of early colonizers from oral cavity. The methods used to evaluate antimicrobial activity include the following: determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in microtiter plates for growth in suspension; and quantification of biomass by crystal violet staining and counting of colony forming units for biofilm growth. The microorganisms were grown in Brain Heart Infusion broth at 37 °C under atmospheric pressure with 5% CO_2. The peptides were solubilized in 0.1% acetic acid (v/v) at various concentrations (500 to 15.6 µg/ml). Chlorhexidine gluconate 0.12% was used as the positive control, and BHI culture medium was used as the negative control. The tested peptide [W^7]*KR12*-KAEK demonstrated a remarkable antimicrobial and antibiofilm effects, with enhanced activity when compared to KR-12 peptide, and also inhibiting the planktonic and biofilm growth of all strains tested. The peptide [W^7]*KR12*-KAEK is a potential biotechnological input for the development of auxiliary antimicrobial therapies for controlling infectious diseases of oral cavity.

Keywords: Antimicrobial Peptide; Dental Carie; Biofilm; Periodontal Disease; Early Colonizers

1. Introduction

The oral cavity is considered one of the most infected sites throughout the body. Large part of the microorganisms that inhabit this site are considered native and beneficial, living in harmony with the host [22]. Thus, due to the high level of interspecies relations found in the oral cavity, the term oral microbiota has been gradually replaced by oral microbiome [4].

The oral microbiome comprises more than 1000 different species, including viruses, bacteria and fungi, each one of them inhabiting different niches of the oral cavity with their respective characteristics [27]. However, a characteristic that all the microorganisms that inhabit the oral cavity possess is the ability to adhere to oral surfaces [17].

This capability is critical for oral microorganisms, since without adherence to tissues, bacteria are carried out from the mouth by salivary flow or into the digestive tract. In this context, oral microorganisms can be classified into two groups: the early colonizers, microorganisms, mostly bacteria which possess the ability to direct adhesion to molecules present on the surface of the oral tissues; and late colonizers, microorganisms that use molecules expressed on the surface of the primary colonizers to attach to the oral structures [17,36,40].

Streptococci are the main bacterial genus that composes the group of early colonizers. This particular genus can recognize a number of molecules present on the acquired enamel pellicle, as statherin, proline-rich proteins, salivary α-amylase, sialylated mucins and salivary agglutinin [17]. The vast majority of these bacteria do not cause diseases to the host, however, when these bacteria proliferate on the surface of tissues and enable the coadhesion of late colonizers, these microorganisms arrange themselves in complex biofilms that may culminate in the appearance of caries, periodontal disease and candidiasis [4,40].

According to Kolenbrander and coworkers, in 2010, in oral pathogenic biofilms, the main streptococci that initially colonize oral surfaces are *S. oralis*, *S. sanguinis* and *S. parasanguinis*. Such species serve as an anchor for other pathogens to develop on the tissue, causing dental caries or periodontal disease [17].

In a previous study, it was demonstrated that strains of *Candida albicans,* a yeast present in the oral cavity, are stimulated to grow within the presence of certain streptococci, such as *S. salivarius* and *S. oralis*. In addition the growth rate, the rates of biofilm formation and secretion of toxins are increased in the simultaneous presence of these species [30,42].

Another species commonly found in carious lesions by its high capacity to produce acids is *Streptococcus sobrinus*. Although it is a species directly related to the appearance of carious lesions, along with *S. mutans*, it has the ability to bind directly to the acquired enamel pellicle also exerting the function of an initial colonizing [16].

Thus, due to the intimate participation of the early colonizers in the initiation of pathogenic biofilms, new therapies to control these microorganisms have been widely studied nowadays [1,8,19].

Antimicrobial peptides are a broad class of molecules produced by various cells and tissues of living beings with the main purpose of controlling microbial colonization within the host. In humans, these peptides are secreted by epithelial cells and cells of the innate immune system not only with the antimicrobial purposes, but also as immunomodulatory molecules [7,12].

Among the different human antimicrobial peptides, we highlight the KR-12 peptide. This molecule is derived from human peptide LL-37 and has been the subject of several studies by having a small size and low tissue toxicity, serving as an ideal model for the search for new molecules with biotechnological purpose [14,25].

Thus, the aim of this study was to evaluate the antimicrobial and antibiofilm activity of a synthetic antimicrobial peptide [W^7]*KR12*-KAEK (KRIVQRWKDFLRKAEK-NH_2), based on the primary structure of the native peptide KR-12, on different strains of early colonizers of oral cavity.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The following bacterial strains: *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 6715, *Streptococcus oralis* ATCC 10557, *Streptococcus salivarius* ATCC 7073 and *Streptococcus parasanguinis* ATCC 903 were grown in Brain Heart Infusion Agar (BHIA, Difco, Himedia, India) for 24 h 37°C and 5% CO_2. After that, isolated colonies were removed and inoculated into 5 ml of sterile BHI broth under the same conditions. Prior to assays, bacterial cells were adjusted to concentration of 10^6 CFU/ml in BHIs for all experiments of antimicrobial and antibiofilm activity.

2.2. Peptide synthesis, purification and preparation

The peptide [W^7]*KR12*-KAEK (KRIVQRWKDFLRKAEK-NH_2) were synthesized using a Protein PS-3 synthesizer by solid phase peptide synthesis using standard 9-fluorenylmethyloxycarbonyl (Fmoc) protocols on Fmoc-Arg(Pbf) [23]. The amino acids were coupled at a four-fold excess using O-Benzotriazole-N,N,N’,N’-tetramethyluronium-hexafluoro-phosphate (HBTU)/N-methylmorpholine

(NMM) (v/v) with N,N-dimethylformamide (DMF). The α-amino group deprotection step was performed in 20% piperidine/DMF for 1 and 20 min. Cleavage of the peptide from the resin and removal of the side chain protecting groups were simultaneously performed with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropyl silane for 2 h. After this procedure, the crude peptide was precipitated with anhydrous ethyl ether, separated from soluble non-peptide material by centrifugation, extracted into 5% solvent B (0.036% (v/v) TFA/acetonitrile) and 95% solvent A (0.045% (v/v) TFA/H_2O) and lyophilized. The crude peptide was purified by semi-preparative HPLC on a Beckman System Gold using a reversed-phase C18 column with a linear gradient of 5–45% solvent B for 90 min. The flow rate was 5 ml/min. Ultraviolet (UV) detection was carried out at 220 nm. The peptide homogeneity was checked by analytical HPLC on a Shimadzu system, using solvents A and B with a linear gradient of 5–95% (v/v) solvent B for 30 min, at a flow rate of 1.0 ml/min and UV detection at 220 nm. The identity of the peptide was confirmed by mass spectrometry in positive-ion mode ESI on a Bruker model apparatus.

The peptide was fully solubilized in sterile deionized water with 0.1% acetic acid (CH_3COOH) at a concentration of 500 μg/ml prior to use and stored in a freezer at -20ºC.

2.3. Antimicrobial activity assay

The antimicrobial activity of [W^7]*KR12*-KAEK was assessed by the microdilution test in 96-well "U" bottom microtiter plates. The plates were prepared with sterile BHI containing the peptide [W^7]*KR12*-KAEK at a concentration of 500 μg/ml. Then, two-fold serial dilutions were made to achieve varying concentrations (7.8 to 500 μg/ml) at a final volume of 100 μl. Next, 100 μl of cells (2 x 10^6 CFU/ml) was added to each well to yield a final volume of 200 μl. Chlorhexidine gluconate (0.12%) and BHI sterile culture medium were added to separate wells as the positive and negative controls, respectively.

The plates were incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the turbidity of each well was measured using a spectrophotometer (SpectraMax® I3) at 620 nm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of inhibiting bacterial growth visually.

To determine the minimum bactericidal concentration (MBC), Petri dishes with BHI agar were inoculated with 10 μl from the wells that showed no visible microbial growth. The MBC was defined as the lowest concentration of [W^7]*KR12*-KAEK capable of completely inhibiting microbial growth on the Petri dishes.

2.4. Antibiofilm activity assay

The assays of biofilm formation were performed according to the methodology described by previous research, in 96-well "flat" bottom microtiter plates with some modifications [37]. The plates were prepared in the same manner as in the antimicrobial activity test. However, two plates were prepared for each test, one for the quantification of biomass (crystal violet) and one for counting the CFU (Colony Forming Units) adhered to the plate.

2.4.1. Quantification of biomass

After 24 hours of incubation at 37°C, the plates were washed three times with sterile distilled water, and then 200 μl of methyl alcohol P.A. was added to fix adhered cells. After the removal of methanol, 200 μl of 1% crystal violet was added for 5 minutes. This procedure was performed to allow an indirect quantification of biofilm formation by staining with crystal violet. Then, the washing procedure was repeated and the plate placed at room temperature for drying. To dissolve the crystal violet bound to the biofilm, 200 μl of 33% acetic acid were added to wells and the absorbance of each well was measured with a spectrophotometer (SpectraMax® I3) at 595 nm.

2.4.2. Count of Colony Forming Units (CFU)

After incubation for 24 hours at 37°C, the plates were washed three times with sterile distilled water to remove weakly attached cells. Next, 200 μl of sterile 0.9% NaCl solution was added to each well of the plate, and the plate was then subjected to an ultrasonic bath (Sonicor/SC-52) for 6 minutes to release the cells from the formed biofilm. The volume of five wells was removed with an up-down movement and collected into a sterile 2.0 ml microtube to a final volume of 1 ml. Ten-fold serial dilutions of the cell suspension were performed for subsequent plating. Then, the plates were incubated at 37°C with 5% CO_2 for 24 hours. The number of colonies grown on each plate were counted and converted to units of CFU/ml (Log_{10}).

2.5. Statistical analysis

All experiments were performed in triplicate, and the results were entered into Microsoft Excel (Version 2010 for Windows) and later analyzed with GraphPad Prism software (version 5.0 for Windows). To analyze the significant differences between groups, one-way ANOVA was conducted with a Bonferroni post-test, and $p<0.05$ was considered significant.

3. Results

3.1. Antimicrobial activity

Despite the peptides KR-12 and [W^7]*KR12*-KAEK be similar to one another, the effects exerted in the tests with planktonic cells were significantly different for all bacterial strains analyzed.

The KR-12 peptide did not exhibit antimicrobial activity against the bacterial strains studied in any of the concentrations used. While the modified peptide [W^7]*KR12*-KAEK demonstrated bactericidal and bacteriostatic activity for all strains in different concentrations. The lowest concentration with bacteriostatic activity was 7.8 μg/ml for *S. salivarius, S. paransaguinis* and *S. sobrinus*, and the lowest concentration with bactericidal activity was 15.6 μg/ml for *S. salivarius* and *S. parasanguinis* (Table 1).

Table 1: *In vitro* susceptibility of oral microorganisms tested against the peptides KR-12 and [W^7]*KR12*-KAEK.

Microorganism	Strain	MIC values	MBC values	MIC values	MBC values

		KR-12 Peptide (µg/ml)	KR-12 Peptide (µg/ml)	[W^7]*KR12*-KAEK (µg/ml)	[W^7]*KR12*-KAEK (µg/ml)
Streptococcus oralis	ATCC 10557	-	-	15.6	31.25
Streptococcus salivarius	ATCC 7073	-	-	7.8	15.6
Streptococcus sanguinis	ATCC 10556	-	-	31.25	31.25
Streptococcus parasanguinis	ATCC 903	-	-	7.8	31.25
Streptococcus sobrinus	ATCC 6715	-	-	7.8	15.6

3.3. Antibiofilm activity

3.3.1. Analysis of quantification of biomass

For biofilms inhibition assays, it was expected that the KR-12 peptide did not exhibit any significant result because of its poor effect on planktonic bacteria. However, surprisingly, on the bacteria *S. parasanguinis* ATCC903, an interference in the formation of biofilms starting at a concentration of 31.25 µg/ml was shown (Figure 1B). At the highest concentration tested, the same peptide was able to decrease by approximately 28% of the total biomass for strain *S. parasanguinis* ATCC903. However, no other activity was noted for the other species (Figures 1A, 1C-E).

As for the modified peptide [W^7]*KR12*-KAEK, its effects on the reduction of biomass production were extremely satisfactory for all strains tested.

The most significant results were obtained on the strains *S. parasanguinis* ATCC903 and *S. sobrinus* ATCC6715 in which, in all concentrations, a significant interference in biofilm formation was detected (Figures 1G and 1J). Moreover, in their minimum bactericidal concentrations, the peptide [W^7]*KR12*-KAEK was able to completely inhibit the formation of biomass of these bacteria.

Regarding the other species tested, all exhibited a complete inhibition in the formation of biomass under the peptide [W^7]*KR12*-KAEK in minimum bactericidal concentration.

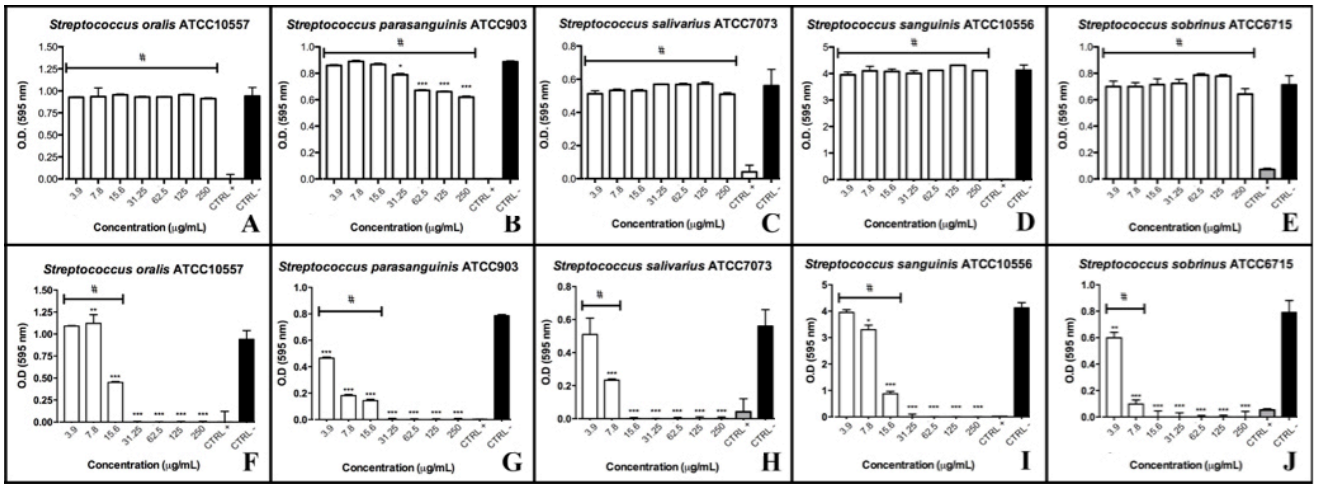

Figure 1: Quantification of biomass for analysis of antibiofilm activity. Effect of KR12 peptide (A-B) and its analogue [W^7]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▨). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

3.3.2. Counts of Colony Forming Units

Based on results of quantification of biomass, the couting of CFU were performed for all concentrations tested for the native peptide KR12 and only at concentrations below the MBC for the peptide [W^7]*KR12*-KAEK because, at higher concentrations, there were no viable colonies to count due to its bactericidal activity (Figure 2).

Following the results previously obtained for quantification of biomass, the antimicrobial effect of KR-12 peptide on the cell count embedded in the biofilm was not encouraging (Figures 2A-E). However, regarding the result of this test for *S. parasanguinis*, it is noted that although the interference in biomass production evidenced above, the amount of viable cells embedded in the biofilm remains the same, except for the concentration of 250 µg/ml which showed slight decrease in the number of cells present in the biofilm (Figure 2B).

Corroborating with the results described above, the peptide [W^7]*KR12*-KAEK was able to completely eliminate the presence of bacterial cells adhered to the well surface at its minimum bactericidal concentration for each species (Figures 2F-J). However, a data obtained deserves better observation. In the results of the strain *S. oralis* ATCC10557, it is clear that, at a concentration of 7.8 µg/ml, the counting of colony forming units was reduced by approximately 64% (Figure 2F). However, when observed the biomass quantification assay in the same concentration to the same bacterial strain (Figure 1F), it is noted that biomass production is exaggerated, and even significantly higher than the negative control. This result suggests that possibly, although few viable cells present in the biofilm, these are under chemical stress situation and increase its polymeric extracellular matrix production rate.

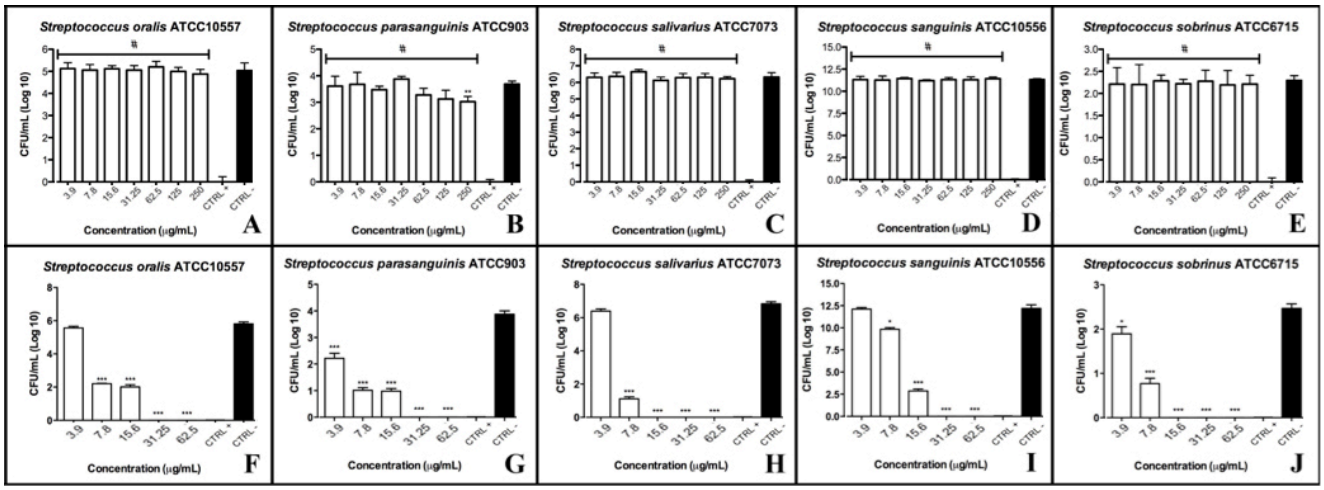

Figure 2: Counting of colony forming units for analysis of antibiofilm activity. Effect of KR12 peptide (A-B) and its analogue [W^7]*KR12*-KAEK (C-D) against the bacterial strains analyzed. Peptide tested (□), Negative control (■) and Chlorhexidine Gluconate 0.12% (▣). *p<0.05; **p<0.01; ***p<0.001 compared to negative control. # p<0.001 compared to positive control.

4. Discussion

Dental caries as well as periodontal diseases, are the oral diseases that most affect patients around the world. According to a recent survey, about 90% of the population is or has been affected by any of these diseases throughout life [26].

Both dental caries as periodontitis have an intrinsic microbiological factor for its development. Thus, they can be considered as infectious diseases. However, the formation of these lesions is usually slow and susceptible to prevention. However, current control methods are highly dependent on cooperation of the patient and the uncontrolled use of oral antibiotics can lead to increasingly less susceptible species to these agents [3,35].

With the scientific advancement in isolation techniques, purification and molecular synthesis, several studies seeking new methods in controlling the oral microbiome have been developed with emphasis on synthetics or natural products [8,19,34].

Within this scope, the antimicrobial peptides (AMPs) emerge as a viable alternative for the treatment and prevention of infectious diseases, due to its low production cost, greater facility in the synthesis process, low toxicity and high biocidal power at low concentrations [6,7].

The use of AMPs against oral bacteria and in particular upon initial colonizers has been previously described in the literature. In a study by Tu and colleagues, in 2016, the synthetic peptide GH12 was able to exert antimicrobial activity against strains of *S. sobrinus* and *S. salivarius* in concentrations between 6.7 - 32.0 µg/ml [39]. This range of activity found by the authors was similar to data found in our research.

Moreover, the application of AMPs are not restricted to the use of the isolated molecule. Study by Kim and colleagues in 2003 showed that certain cationic peptides can be used in synergy with commercial compounds such as chlorhexidine gluconate and still remain stable against strains of *S. sobrinus*, *S. sanguis* and *S. gordonii* [15].

The peptides used in this study were the human peptide KR-12, derived from cathelicidin LL-37 peptide, and the peptide [W^7]*KR12*-KAEK, novel peptide constructed from the primary structure of KR-12.

The scientific literature previously demonstrates the antimicrobial capacity of the KR-12 peptide against a range of Gram-negative bacteria [14,25]. However, there is little about its effect on Gram-positive bacteria. As expected, this molecule was not effective in controlling the bacteria analyzed, both in planktonic state and in biofilms.

Nevertheless, the modifications to the primary structure of KR-12 were effective in increasing its spectrum of action. Such activity is probably due to addition of certain amino acids at specific locations along the sequence. The addition of positively charged amino acids at the N-terminal region of the peptide may have influenced the increase of the antimicrobial activity. It is known que the positive charge on cationic peptides is able to directly change its biocide effect [5,32]. Moreover, Tryptophan proved to be important for the peptide interaction with the membrane surface. In a previous study, it was found the prevalence of certain amino acids in many antimicrobial peptides. Of all the amino acids, Tryptophan was the most frequent, and even the most associated with high antimicrobial activity of the peptides. These findings are probably due to the hydrophobic character of this amino acid, influencing the relationship between hydrophobicity and charge of the antimicrobial peptide [24].

The present study evaluated the antimicrobial and antibiofilm activities of two antimicrobial peptides on oral bacteria. Bacteria denominated as early colonizers of oral biofilms are extremely important for the development of pathological processes linked to biofilms [18].

Among the early colonizers, *Streptococcus parasanguinis* plays a key role in the development process of cariogenic biofilm. Such a role is played by a series of genes related to the production of binding proteins, such as Fap1 gene in charge of producing the fimbriae responsible for adherence of the microorganism itself to the tooth surface [10,11]; and BaP1 gene, responsible for the direct linkage between initial and secondary colonizers, contributing to the development of the biofilm [20]. Thus, we highlight the remarkable antimicrobial and antibiofilm activity of the peptide [W^7]*KR12*-KAEK on these species, which gives broad applicability in the control of oral biofilms in its initial formation stage.

Another microorganism analyzed in this research that deserves attention is the *S. oralis*. This microorganism is closely linked to the initial process of biofilm formation and with the facilitation of adhesion mechanisms to periodontal bacteria, such as *Porphyromonas gingivalis* [21]. Still regarding the *S. oralis*, it has a very versatile gene repertoire. In previously published study, the authors demonstrated the gene modulation in form of plasmids in a dependent manner on the dosage of antibiotics to the culture medium. It was also noticed that genes related to cellular stress such as polymeric extracellular matrix production are overexpressed in these situations [38]. This finding confirms the unique result in the antibiofilm assay performed with [W^7]*KR12*-KAEK, in which, despite the increase in biomass, there was a reduced amount of viable cells within the biofilm, indicating that under conditions stress, these viable cells increased the production of extracellular polymeric matrix.

Another bacterial strain that showed a significant susceptibility to [W^7]*KR12*-KAEK peptide was *Streptococcus sobrinus*. This result deserves attention due to the undeniable role of this species in the process of demineralization of tooth tissue [28]. Although much attention is given to the *S. mutans* because its aciduric and acidogenic skills, current studies support that other bacteria, among them *S. sobrinus*, act in mutualism enhancing and accelerating the dental demineralization process [13]. Furthermore, *S. sobrinus* have the ability to withstand environments with low pH and to produce enzymes that help directly the biofilm resistance to antimicrobial agents and environmental aggressive factors [29].

Finally, it is known that the mechanisms of action of AMPs is not fully elucidated. One of these suggested mechanisms is through direct interaction of the peptide with the bacterial cell membrane and its gradual internalization forming pores that allow the leakage of cytoplasmatic fluid and bacterial structural change [2,31]. In addition, studies show the activity of certain antimicrobial peptides in bacterial metabolic pathways such as gene expression and protein synthesis [9,33,41].

5. Conclusion

It can be concluded, therefore, that the synthetic peptide [W^7]*KR12*-KAEK has increased antimicrobial activity and antibiofilm when compared to the native peptide KR-12. Thus, because of its effectiveness in reducing biofilm formation of initial colonizing bacteria, this peptide stands as biotechnological input in dentistry.

However, more studies are necessary to ensure its safety for clinical use and stability in the oral environment, as well as establishing its specific mechanism of action.

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Capítulo VI

Patente do Peptídeo [W7]-KR12-KAEK

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: UNIVERSIDADE FEDERAL DO CEARÁ

1.2 Qualificação: INSTITUIÇÃO DE ENSINO SUPERIOR

1.3 CNPJ/CPF: 07272636000131

1.4 Endereço Completo: AV DA UNIVERSIDADE , 2853 BENFICA FORTALEZA

1.5 CEP: 60020-180

1.6 Telefone: 8533669434 1.7 Fax:

1.8 E-mail: javam@ufc.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

" PEPTÍDEO ANTIMICROBIANO SINTÉTICO E USO SOBRE BACTÉRIAS ORAIS "

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponivel)	Data de depósito

☐ continua em folha anexa

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: EDSON HOLANDA TEIXEIRA

6.2 Qualificação: DOUTOR

6.3 CPF: 71755985304

6.4 Endereço Completo: RUA MONSENHOR FURTADO S/N ,RODOLFO TEOFILO FORTALEZA CE

6.5 CEP: 60441750

6.6 Telefone: 8533668309 6.7 FAX:

6.8 E-mail: edson@ufc.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐

Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

9.5 CEP:

9.6 Telefone: 9.7 FAX:

9.8 E-mail:

☐ continua em folha anexa

10. Listagem de sequências biológicas.

Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 3/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	01
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	10
☒	11.6	Reivindicações.	02
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, 01 por melhor representar a invenção (sujeito à avaliação do INPI).	02
☒	11.8	Resumo.	01
☐	11.9	Listagem de sequências em arquivo eletrônico: _____nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) CONT DE INVENTORES, LISTAGEM DE SEQUÊNCIAS, CODIGO DE CONTAGEM E UM CD	04

12. Total de folhas anexadas: 20 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

FORTALEZA, 02 DE JUNHO DE 2016

Local e Data

Assinatura e Carimbo

Prof. Henry de Holanda Campos
Reitor da UFC

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO CEARÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COORDENADORIA DE INOVAÇÃO TECNOLÓGICA

DECLARAÇÃO

Declaramos, para os devidos fins, que Bruno Rocha da Silva, é um dos inventores da patente intitulada **"Peptídeo antimicrobiano sintético e uso sobre bactérias orais "**, em depósito no INPI (Instituto Nacional de Propriedade Industrial), sob número de processo de patente definitivo BR 10 2016 013695 4.

Fortaleza, 24 de junho de 2016.

Prof. Antônio Gomes de Souza Filho
Pró-Reitor de Pesquisa e Pós-Graduação

Prof. Antonio Gomes de Souza Filho
Pró-Reitor de Pesquisa e
Pós-Graduação
Universidade Federal do Ceará

Anexo I

Produção científica 2013-2016

Peptides 42 (2013) 78–83

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Peptides

journal homepage: www.elsevier.com/locate/peptides

Antimicrobial activity of the synthetic peptide Lys-a1 against oral streptococci

Bruno Rocha da Silva[a], Victor Aragão Abreu de Freitas[a], Victor Alves Carneiro[d], Francisco Vassiliepe Sousa Arruda[a], Esteban Nicolás Lorenzón[b], Andréa Silvia Walter de Aguiar[c], Eduardo Maffud Cilli[b], Benildo Sousa Cavada[d], Edson Holanda Teixeira[a,*]

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ABSTRACT

The peptide LYS-[TRP^6]-Hy-A1 (Lys-a1) is a synthetic derivative of the peptide Hy-A1, initially isolated from the frog species *Hypsiboas albopunctatus*. According to previous research, it is a molecule with broad antimicrobial activity. The objective of this study was to evaluate the antimicrobial activity of the synthetic peptide Lys-a1 (KIFGAIWPLALGALKNLIK-NH_2) on the planktonic and biofilm growth of oral bacteria. The methods used to evaluate antimicrobial activity include the following: determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in microtiter plates for growth in suspension and quantification of biomass by crystal violet staining and counting of colony forming units for biofilm growth. The microorganisms *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus salivarius*, *Streptococcus mutans* and *Streptococcus sobrinus* were grown in Brain Heart Infusion broth at 37 °C under atmospheric pressure with 10% CO_2. The peptide was solubilized in 0.1% acetic acid (v/v) at various concentrations (500–1.9 μg mL^{-1}). Chlorhexidine gluconate 0.12% was used as the positive control, and BHI culture medium was used as the negative control. The tested peptide demonstrated a remarkable antimicrobial effect, inhibiting the planktonic and biofilm growth of all strains tested, even at low concentrations. Thus, the peptide Lys-a1 is an important source for potential antimicrobial agents, especially for the control and prevention of microbial biofilms, which is one of the most important factors in cariogenic processes.

1. Introduction

Dental caries results from the localized destruction of dental hard tissues susceptible to acidic products from the bacterial fermentation of carbohydrates [36]. It is one of the primary preventable infectious diseases in pediatric patients, although individuals may be affected by dental caries throughout life [8,28,33].

Caries is the major cause of tooth loss and orofacial pain, although it can be controlled on its early stages. However, it is not a self-limiting disease. If therapeutic measures are not taken, it can progress until complete destruction of the tooth [34,40].

A primary etiological factor related to the development of dental caries is the colonization of pathogenic microorganisms on oral surfaces [21]. Such microorganisms vary according to the development of the lesion and some host factors. However, the ability of these colonizers to develop into biofilms is one of the most important virulence factors and, consequently, one of the most studied field [20].

Biofilms are complex microbial communities that develop on a wide variety of surfaces. They are generally associated with an extracellular matrix composed of various types of biopolymers derived from bacterial metabolism [1,11].

Growth in biofilms provides protection against antibiotics and environmental stress factors in two ways: the physical barrier formed by the extracellular matrix, which hinders the penetration of antimicrobial agents into the biofilm [12]; and bacterial communication mechanisms that stimulate bacteria to produce enzymes and proteins important for the physiological adaptation of biofilm [10].

Due to its public health importance as the leading cause of orofacial infections, several studies have been conducted on combating biofilm development with the goal of controlling pathogenic oral microflora [5,7,35,41].

In this context, a group of molecules with unique characteristics, antimicrobial peptides (AMPs), have been the focus of research in the field of microbiology. AMPs are a heterogeneous group of

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ORIGINAL ARTICLE

Profile of brazilian dental students and its relationship to anxiety levels

Perfil dos alunos de odontologia e sua relação com os níveis de ansiedade

Nayane Cavalcante Ferreira da Silva[1]. Bruno Rocha da Silva, M.Sc[2]. Hélvia Menezes Vasconcelos[3]. Patrícia Maria Costa de Oliveira, M.Sc[4]. Edson Holanda Teixeira, Ph.D[5]. Andréa Silvia Walter de Aguiar, Ph.D[6].

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ABSTRACT

The aim of this study was to evaluate the profile of dental students and its relationship to anxiety levels. The State-Trait Anxiety Inventory was applied to 207 first- to fifth-year undergraduates enrolled in the dentistry program at the School of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Brazil. Participants were aged 17 to 29 years, and the maiority were women (57.5%). Students had a weekly workload of up to 40 hours and performed at least two complementary activities, in addition to their undergraduate training. Almost all students (95.7%) had attended private high schools and 80.6% lived with their parents or spouses. Regarding parental education, 59.4% of mothers and 55.6% of fathers had a higher education degree. Most students showed medium levels of anxiety according to measurements of state anxiety (53.1%) and trait anxiety (81.6%). It can be concluded that more than 50% of students in the Dentistry graduation course of the Federal University of Ceará showed an average level of anxiety, and that the excessive workload and enrichment activities necessary to obtain the undergraduate degree in Dentistry probably can be influencing the anxiety levels of students, however, more studies and statistical tests should be performed to identify the root causes in order to preserve the mental health of these future dentists.

Keywords: Dentistry. Dental students. Anxiety. Test anxiety scale.

RESUMO

O objetivo deste estudo foi avaliar o perfil dos estudantes de odontologia e sua relação com os níveis de ansiedade. O Inventário de Ansiedade Traço-Estado foi aplicado a 207 alunos do primeiro ao quinto ano, matriculados no curso de Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará. Os participantes tinham entre 17 a 29 anos, e a maioria eram mulheres (57,5%). Os alunos tiveram uma carga de aulas semanais de até 40 horas e realizaram pelo menos duas atividades complementares, além do curso de graduação. Quase todos os alunos (95,7%) tinham frequentado escolas privadas e 80,6% viviam com seus pais ou cônjuges. Em relação à escolaridade dos pais, 59,4% das mães e 55,6% dos pais tinham um diploma de Ensino Superior. A maioria dos estudantes apresentaram níveis médios de ansiedade, ansiedade-estado (53,1%) e ansiedade-traço (81,6%). Pode-se concluir que mais de 50% dos alunos que frequentam o curso de Odontologia da Universidade Federal do Ceará demonstraram um nível médio de ansiedade, e que a carga horária excessiva e as atividades complementares necessárias para obtenção do título de graduação em Odontologia provavelmente podem estar influenciando os níveis de ansiedade dos alunos, entretanto, mais estudos e testes estatísticos devem ser realizados para identificar as principais causas, a fim de preservar a saúde mental desses futuros cirurgiões-dentistas.

Palavras-chave: Odontologia. Estudantes de odontologia. Ansiedade. Escala de ansiedade frente a teste.

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Stress distribution on maxillary central incisor under similar traumatic situations with different loading forces: a 3-D finite element analysis.

Distribuição de estresse em incisivo central superior sob situações traumáticas semelhantes com diferentes cargas: uma análise tridimensional de elementos finitos.

Bruno Rocha da Silva[1], Nayane Cavalcante Ferreira[2] , José Jeová Siebra Moreira-Neto[3], Francisco Ilson da Silva Jr[4], Edson Holanda Teixeira[1], Andréa Silvia Walter de Aguiar[3]

ABSTRACT

Aim: The present study aimed to analyze the stress distribution in the dentoalveolar structures of a maxillary central incisor submitted to two situations of impact loading. **Materials and Methods:** The following loading forces were applied using a three-dimensional finite element model: a force of 500 N at an angle of 45° on the buccal surface of the crown and a 2000 N force acting in the same direction and surface of the tooth. **Results:** Harmful stress was observed in the second situation, suggesting damage to both the tooth and adjacent tissue. However, the damage found in soft tissues such as dental pulp, was negligible. **Conclusion:** Injuries resulting from the traumatic situations were more damaging to the integrity of the tooth and its associated hard-tissue structures.

Uniterms: Tooth injuries. Finite element analysis. Biomechanics. Computing methodologies. Three-dimensional imaging.

INTRODUCTION

Dental trauma is one of the main emergencies in dentistry. However, the precise biomechanical characteristics of dental trauma and their repercussions on adjacent tissues are largely unknown with little experimental evidence.[1-3]

Dentoalveolar joint (DAJ) injuries are caused by the sum of the effects of impact applied to the tooth. These effects can be direct when the consequences of trauma occur in the area of impact, such as tooth fractures, or indirect when the stress produced by the impact propagates to adjacent tissues and causes damage to these regions, such as root resorption resulting from periodontal ligament (PDL) necrosis.[4,5]

Several methods have been developed in an attempt to better understand the stress distribution inside the dentoalveolar joint (DAJ). Such methodologies include photoelastic models, analytical mathematical models, and mathematical analyses, such as the finite element method (FEM).[6-8]

In FEM, the behavior of a particular physical system is mathematically simulated. A continuous structure is divided into different elements, which maintain the properties of the original structure. Each of these elements is described by differential equations and solved using mathematical models selected according to the data under investigation.[9,10]

FEM allows the researcher to create models for complex structures, reproducing the irregular geometries of either natural or artificial tissues, e.g. the dentoalveolar articulation. In addition, FEM allows one to modify the parameters of those geometries, which makes it possible to apply a force or a system of forces to any point and/or in any direction, thereby providing information on movement and on the degree of tension and compression forces caused by these loads.[8,11]

The aim of this study was to analyze the stress distribution in the dentoalveolar structures of a maxillary central incisor submitted to two similar situations of traumatic impact with different loading forces.

MATERIALS AND METHODS

Three-dimensional geometrical model design

The methodology used to obtain three-

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2013; 25(3): 254-60, set-dez

ASYMPTOMATIC ANTROLITH IN MAXILLARY SINUS. REPORT OF A CASE.

ANTRÓLITO ASSINTOMÁTICO NO SEIO MAXILAR. RELATO DE CASO

Ariel Valente Bezerra*
Manoel de Jesus Rodrigues Mello**
Rodolfo Cavalcante Lira***
Daniel Ximenes da Silveira****
Gabriel Silva Andrade*****
Bruno Rocha da Silva******
Andréa Sílvia Walter de Aguiar*******

ABSTRACT
Antroliths are depositions composed of minerals, such as calcium phosphate, located around a foreign body into the sinuses; the maxillary sinus is most affected by antroliths, followed by the frontal sinus. The aim of this study was to report the case of the patient JVS, a 63-year-old male with no health disorders who was referred to the oral and maxillofacial surgery department of a reference hospital in Fortaleza, CE, Brazil, as a victim of a motorcycle accident. On physical examination, it was found that the patient exhibited fracture of the left maxillary and zygomatic bones. Upon examination by computed tomography imaging, besides the fracture lines, a hyperdense area of well-defined limits in the left maxillary sinus was observed. In surgical treatment, after fixation of facial fractures, a Caldwell-Luc access without lower meatal antrostomy was performed for foreign body removal and sinusectomy with restoration of sinus drainage. The foreign body was sent for histopathological study, which suggested the presence of an exogenous antrolith of the left maxillary sinus. Thus, it can be concluded that a careful analysis of imaging tests may show unusual changes found in the antral cavity, even without the occurrence of any clinical symptoms.
DESCRIPTORS: Surgery, oral; Paranasal sinuses; Foreign bodies

RESUMO
Antrólitos são constituídos de deposições minerais como o fosfato de cálcio em torno de um corpo estranho dentro dos seios paranasais, dentre os quais o seio maxilar constitui-se o mais acometido, seguido do seio frontal. O objetivo do presente trabalho é relatar o caso do paciente J.V.S., sexo masculino, 63 anos, normossistêmico, vítima de atropelamento motociclístico, que foi encaminhado para o serviço de cirurgia e traumatologia bucomaxilofacial de um hospital de referência em Fortaleza, CE, Brasil. Ao exame físico, constatou-se que o paciente portava fratura dos ossos maxilar e zigomático esquerdos. Ao exame imaginológico por tomografia computadorizada, além das linhas de fraturas, foi visualizada uma área hiperdensa de limites bem definidos em seio maxilar esquerdo. No tratamento cirúrgico, após a fixação das fraturas faciais, foi realizado acesso de Caldwell-Luc sem antrotomia meatal inferior para remoção do corpo estranho e sinusectomia com restabelecimento da drenagem sinusal. O corpo estranho foi enviado para estudo histopatológico que apresentou laudo sugestivo de antrólito exógeno no seio maxilar esquerdo. Dessa forma, pode-se concluir que a análise criteriosa dos exames de imagem pode evidenciar alterações incomuns encontradas nas cavidades antrais, mesmo sem a ocorrência de nenhuma sintomatologia clínica.
DESCRITORES: Cirurgia bucal, Seios paranasais, Corpos estranhos

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** Mestre em Cirurgia e Doutorando em Odontologia: area de concentração em Cirurgia pela Universidade Federal do Ceará (UFC), Chefe do Serviço de Cirurgia e Traumatologia Bucomaxilo Faciais – Instituto Dr. José Frota (IJF), Fortaleza/CE, Brasil. Email: mjrmello@gmail.com
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****** Mestre em Biotecnologia e Doutorando em Biotecnologia – RENORBIO/ Universidade Federal do Ceará (UFC), Fortaleza/CE, Brasil. Email: brunorocha747@gmail.com
******* Professora Adjunto do Departamento de Clínica Odontológica da Faculdade de Farmácia, Odontologia e Enfermagem (FFOE) da Universidade Federal do Ceará (UFC), Fortaleza/CE, Brasil. Email: andrea.aguiar@ufc.br

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BioMed Research International
Volume 2014, Article ID 729358, 7 pages
http://dx.doi.org/10.1155/2014/729358

Research Article

Antimicrobial Effect of the Triterpene 3β,6β,16β-Trihydroxylup-20(29)-ene on Planktonic Cells and Biofilms from Gram Positive and Gram Negative Bacteria

Francisco Flávio Vasconcelos Evaristo,[1] Maria Rose Jane R. Albuquerque,[2] Hélcio Silva dos Santos,[2] Paulo Nogueira Bandeira,[2] Fábio do Nascimento Ávila,[2] Bruno Rocha da Silva,[1] Ariana Azevedo Vasconcelos,[1] Érica de Menezes Rabelo,[1] Luiz Gonzaga Nascimento-Neto,[1] Francisco Vassiliepe Sousa Arruda,[1] Mayron Alves Vasconcelos,[1,3] Victor Alves Carneiro,[1] Benildo Sousa Cavada,[3] and Edson Holanda Teixeira[1]

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Academic Editor: Pascal O. Bessong

This study evaluated the antimicrobial effect of 3β,6β,16β-trihydroxylup-20(29)-ene (CLF1), a triterpene isolated from *Combretum leprosum* Mart., in inhibiting the planktonic growth and biofilms of Gram positive bacteria *Streptococcus mutans* and *S. mitis*. The antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antibiofilm potential was determined by quantifying total biomass and enumerating biofilm-entrapped viable bacteria. In addition, the acute toxicity of CLF1 on *Artemia* sp. nauplii was also determined. The results showed that CLF1 was able in inhibiting the growth of *S. mutans* and *S. mitis* with MIC and MBC of 7.8 μg/mL and 15.6 μg/mL, respectively. CLF1 was highly effective on biofilms of both bacteria. Only 7.8 μg/mL CLF1 was enough to inhibit by 97% and 90% biomass production of *S. mutans* and *S. mitis*, respectively. On the other hand, such effects were not evident on Gram negative *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. The toxicity tests showed that the LC_{50} of CLF1 was 98.19 μg/mL. Therefore, CLF1 isolated from *C. leprosum* may constitute an important natural agent for the development of new therapies for caries and other infectious diseases caused by *S. mutans* and *S. mitis*.

1. Introduction

The resistance to available antimicrobials is currently a public health concern in the world. Several types of infectious diseases have become difficult to treat and expensive to cure mainly due to the low efficiency of antimicrobials for current bacteria [1, 2]. Moreover, the socioeconomic impact caused by infectious diseases is quite significant as shown by the high amount of financial resources spent on the clinical treatment of patients [3, 4]. Taking this into consideration, the ability of microorganisms to grow as biofilms and the increased rate of microbial resistance to conventional antibiotics contribute to the expanding epidemiology of infectious diseases [5].

Biofilms are complex microbial communities that establish themselves on a wide variety of surfaces and are generally associated with an extracellular matrix consisting of different

Anexo II

Normas das Revistas Científicas

ARCHIVES OF ORAL BIOLOGY

A Multidisciplinary Journal of Oral & Craniofacial Sciences

AUTHOR INFORMATION PACK

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ISSN: 0003-9969

DESCRIPTION

Archives of Oral Biology operates a web-based submission and review system. Please register at http://ees.elsevier.com/aob to submit a paper.

Archives of Oral Biology is an international journal which aims to publish papers of the highest scientific quality in the **oral** and **craniofacial** sciences. The journal is particularly interested in research which advances knowledge in the mechanisms of **craniofacial development** and **disease**, including: **Cell** and **molecular biology Molecular genetics Immunology Pathogenesis Cellular microbiology Embryology Syndromology Forensic dentistry** The aim is to be inclusive and multidisciplinary and papers are also welcome in the fields of structure and function of craniofacial tissues over the whole range of vertebrates including studies concerned with palaeontology and comparative anatomy. *Archives of Oral Biology* will also publish expert reviews and articles concerned with advancement in relevant methodologies. The journal will only consider clinical papers where they make a significant contribution to the understanding of a disease process.

AUDIENCE

Oral biologists, physiologists, anatomists, pathologists.

IMPACT FACTOR

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ABSTRACTING AND INDEXING

Abstracts in Anthropology
Abstracts on Hygiene and Communicable Diseases
Agris
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• immunology
• pathogenesis
• microbiology
• biology of dental caries and periodontal disease
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Archives of Oral Biology will also publish expert reviews and articles concerned with advancement in relevant methodologies. The journal will only consider clinical papers where they make a significant contribution to the understanding of a disease process.

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↑

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