UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba
Faculdade de Odontologia

PAULA FERNANDA KRELING DOMINGUES

Avaliação do potencial antimicrobiano e citotoxicidade de fragmentos peptídicos catiônicos isolados ou combinados na prevenção da cárie dentária

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Tese apresentado à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como parte dos requisitos para a obtenção de título de Doutor em Ciência Odontológica - Área de Concentração: Saúde Bucal da Criança.

Orientadora: Profa. Dra. Cristiane Duque

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À Deus

Meu criador

Sempre em primeiro lugar

À minha família

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Aos meus queridos pais

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RESUMO

O sistema imune tem diversas formas de defesa contra microrganismos patogênicos. As membranas da mucosa são uma fonte de peptídeos catiônicos antimicrobianos contra uma ampla variedade de bactérias, fungos e vírus encapsulados. O objetivo deste estudo foi avaliar a citotoxicidade e atividade antimicrobiana em condições planctônicas e de biofilme de fragmentos derivados de peptídeos catiônicos (PC): LL-37 (originário de hCAP-18), D6-17 e D1-23 (originários de ortólogo da β-defensina-3 humana) contra bactérias cariogênicas. Para análise citotóxica, duas linhagens de células epiteliais foram expostas a diluições seriadas de fragmentos de PC. Ensaios de MTT e coloração de DAPI foram realizados para avaliar o metabolismo e a morfologia celular, respectivamente. A concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) foram determinadas para fragmentos de PC e controle (digluconato de clorexidina - CHX) contra *Streptococcus mutans (Sm), S. mitis, S. oralis, S. salivarius, S. sanguinis, Lactobacillus acidophilus, L. casei, L. rhamnosus, L. brevis, L. fermentum* e *Actinomyces israelii*. A concentração inibitória fracionada (FIC) foi obtida pelas combinações de fragmentos de CP para *S. mutans.* O ensaio de biofilme foi conduzido com CHX e o melhor fragmento de CP contra cepas de *S. mutans*. Microscopia confocal a laser foi realizada para avaliar a quantidade de células mortas em relação as células vivas e também a espessura do biofilme. Os resultados indicaram que D6-17 não afetou o metabolismo de nenhuma das linhagens celulares. D1-23, LL-37 e CHX não foram tóxicos para ambas as células utilizadas, quando de concentrações abaixo de 0,2, 0,02 e 0,01mM, respectivamente. Combinações dos PC não mostraram efeito sinérgico contra *S. mutans.* A coloração DAPI demonstrou fragmentação nucleica para LL-37 e CHX, e aspecto semelhante ao controle (meio de cultura) para D1-23 e D6-17. D1-23 apresentou a melhor atividade bactericida contra *S. mutans, S. mitis* e *S. salivarius.* LL-37 apresentou melhor efeito contra espécies de *Lactobacillus* e

Actinomyces. D6-17 mostrou atividade bactericida apenas contra *S. mutans, L. brevis* e *L. fermentum.* Combinações de fragmentos de PC não mostraram efeito sinérgico contra *S. mutans.* D1-23 (10x CBM) apresentou atividade contra biofilme de *S. mutans* superior a CHX. A microscopia confocal mostrou alta taxa de células mortas em relação a células vivas para D1-23 e CHX quando comparado ao grupo controle (meio de cultura). D1-23 também diminuiu a espessura do biofilme em relação ao grupo controle. Conclui-se que D1-23 mostrou relevante atividade antimicrobiana/antibiofilme contra bactérias cariogênicas e baixa toxicidade em células epiteliais.

Palavras-chave: Cárie Dentária; Peptídeos; Testes de Sensibilidade Microbiana; Células cultivadas.

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ABSTRACT

The immune system has several forms of defense against pathogenic microorganisms. The mucous membranes are a source of potent antimicrobial cationic peptides against a broad range of bacteria, fungi and enveloped viruses. The aim of study was evaluated the cytotoxicity and antimicrobial activity under planktonic and biofilm conditions of fragments derived from cationic peptides (CP): LL-37 (from hCAP-18), D6-17 and D1-23 (from β-defensin-3 derivative) against cariogenic bacteria. For cytotoxicity analysis, two lines of epithelial cells were exposed to serial dilutions of the CP fragments. MTT assays and DAPI staining were performed to evaluate cell metabolism and morphology, respectively. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the CP fragments and control (Chlorhexidine digluconate-CHX) against *Streptococcus mutans (Sm), S. mitis, S. oralis, S. salivarius, S. sanguinis, Lactobacillus acidophilus, L. casei, L. rhamnosus, L. brevis, L. fermentum and Actinomyces israelii*. Fractional inhibitory concentration (FIC) was obtained for the combinations of CP fragments on *S. mutans.* Biofilm assays were conducted with CHX and the best antimicrobial CP fragment against *S. mutans* strains. Confocal Laser Scanning Microscopy (CLSM) was used to analyze the Live/Dead cells and biofilm thickness. The results indicated that D6-17 did not affect the metabolism of either cell line. D1-23, LL-37 and CHX were not toxic for both cells, in concentrations below 0.2, 0.02 and 0.01mM, respectively. DAPI-staining cells demonstrated nuclei fragmentation for LL-37 group and cells with the aspect of apoptosis in the CHX group. Cells treated with D1-23 and D6-17 presented morphology similar to the control group (culture medium). D1-23 presented the best bactericidal activity against *S. mutans, S. mitis* and *S. salivarius.* LL-37 had a better effect against *Lactobacillus* and *Actinomyces* species. D6-17 showed bactericidal activity only against *S. mutans, L. brevis* and *L. fermentum.* Combinations of CP fragments did not show a synergic effect against *S. mutans*. D1-23 (10x MBC) presented a higher effect against *S. mutans* biofilm

compared to CHX. CLSM analysis showed that D1-23 and CHX groups presented higher quantification of dead cells when compared to control (culture medium). The biofilm thickness were lower in the D1-23 group compared to CHX and control groups. We concluded that D1-23 showed a remarkable antimicrobial/anti-biofilm effect against cariogenic bacteria and low toxicity for epithelial cells.

Keywords: Dental Caries; Peptides; Microbial Sensitivity Tests; Cell Culture.

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Sumário

Introdução Geral

A cárie é uma doença infecciosa, causada por ácidos provenientes da fermentação microbiana dos carboidratos da dieta que, com o tempo, causam a desmineralização dos tecidos duros do dente (Fejerskov e Kidd, 2005). Apesar de haver um declínio de sua prevalência no mundo, a cárie dentária continua sendo a doença infecciosa crônica mais comum em crianças, tanto em países em desenvolvimento, quanto nos desenvolvidos (Vadiakas, 2008; Colak et al., 2013). Se não tratada, pode trazer diversos problemas para as crianças, incluido dor (Lygidakis et al., 1998), diminuição na alimentação, maloclusão e até mesmo problemas em seu desenvolvimento (Isong et al., 2014). Quando atinge crianças muito jovens, a partir da erupção dos primeiros dentes decíduos é denominada cárie precoce da infância – CPI (Drury et al., 1999). No Brasil, a CPI apresenta-se como grave problema de saúde pública, com prevalência de 26,5% em crianças com 5 anos de idade (SBBrasil, 2010). Para facilitar o diagnóstico clínico e a padronização terminológica, foi estabelecido que a CPI é caracterizada pela presença de uma ou mais superfícies dentárias cariadas, cavitadas ou não, perdidas ou restauradas, em menores de 6 anos (American Academic of Pediatric Dentistry, 2009). Devido à rápida destruição dentária em curto período de tempo, tem sido sugerida a associação entre os seguintes fatores na etiologia da CPI: ingestão frequente de dieta rica em carboidratos fermentáveis como a sacarose, altas contagens de estreptococos mutans e maior vulnerabilidade imunológica da criança (Mattos-Graner et al., 1998; Mohebbi et al., 2008). Por isso, medidas preventivas e buscas por alternativas que venham amenizar esse quadro são fundamentais para a saúde e bem estar das crianças (Isong et al., 2014).

O grupo bacteriano considerado mais cariogênico é o dos estreptococos mutans (SM), especialmente *Streptococcus mutans* (Mattos-Graner et al., 2001; van Houte et al., 1991). Embora a associação entre SM e CPI pareça convincente, grande percentual das crianças colonizadas por essa espécie bacteriana não manifestam a doença (Mattos-Graner et al., 1998; Ramos-Gomes et al., 2002). Estudos têm mostrado que características genotípicas e fenotípicas do *S. mutans* podem determinar o estabelecimento da espécie no biofilme dental e sua cariogenicidade (Duque et al. 2009; Saxena et al., 2008; Lemos et al., 2005). Assim, outras espécies acidogênicas e acidúricas, incluindo estreptococos não mutans, *Actinomyces* estão envolvidas com o início das lesões de cárie (Sansone et al., 1993; van Houte et al., 1994). Takahashi e

Nyvad (2011) concluíram que muitos microrganismos são igualmente acidogênicos e acidúricos, incluindo espécies de *Actinomyces* e *Lactobacillus*, ambos com importante papel no desenvolvimento do processo carioso. *Lactobacillus* estão principalmente envolvidos na progressão da cárie dentária, uma vez que tem sido encontrado em lesões cariosas avançadas em dentina (Becker et al., 2002). *Actinomyces* é considerado um colonizador inicial, com importante papel no desenvolvimento do biofilme dental (Arai et al., 2015) contribuindo para a colonização de outras espécies bacterianas (Palmer et al., 2003), além disso, também está ligado ao aparecimento da lesão de cárie na superfície radicular (van Houte et al., 1994).

Para auxiliar na defesa contra microbiota patogênica o sistema imunológico humano conta com diversas formas de defesa. As mucosas, além de apresentarem a função de barreira física contra a entrada de organismos estranhos, são fontes de potentes peptídeos catiônicos antimicrobianos (PCAM). Estes apresentam ação contra uma ampla variedade de bactérias, fungos e vírus encapsulados e promovem modulação da resposta imune do hospedeiro, mantendo a microbiota normal em estado estável em diferentes nichos, como a pele, os intestinos e a cavidade bucal (Mccormick e Weinberg, 2010; Wiesner e Vilcinskas, 2010). Entre os principais PCAM presentes na saliva e também no fluido crevicular estão as defensinas e catelicidinas.

As defensinas são peptídeos pequenos, de 15 a 45 aminoácidos, que dependendo do padrão de pareamento de seus resíduos de cisteína, são subdivididas em duas principais subfamílias: α e β-defensinas, ambas apresentam função imunomoduladora, modificando a migração e maturação celular, induzindo citocinas e a liberação de histamina e prostaglandina A2 de mastócitos (Abiko et al., 2003; Mccormick e Weinberg, 2010). Além disso, esses peptídeos vêm demonstrando potente atividade antimicrobiana contra um amplo espectro de patógenos, incluindo os bucais (Ouhara et al., 2005). As α-defensinas são produzidas principalmente pelos neutrófilos, peptídeo neutrofílico humano (HNP-1 a 4), e células de Paneth, defensinas humanas 5 e 6 (HD5 and HD6) (Jones e Bevin, 1992; Chairatana et al., 2016; Nakamura et al., 2016). As β-defensinas (hBDs-1 a 3) são produzidas predominantemente no epitélio (Dale e Fredericks, 2005), superfície da mucosa e trato reprodutivo, têm demostrado um forte espectro antimicrobiano *in vitro*, além de ser quimioatrativos para várias células do sistema imune (Yang et al., 1999; Wu et al., 2003; Dietrich et al.,

2014). Reynolds et al. (2010) avaliaram a atividade antibacteriana de fragmentos de β-defensina 3 e encontraram que a metade N-terminal do aminoácido 23 (D1-23) do Defb14-1CV (ortólogo de rato da β-defensina 3 humana) é um potente agente antimicrobiano.

A catelicidina (hCAP-18) é um peptídeo catiônico α-helical sem cisteína (Bals; Wilson, 2003). É o único desse gênero encontrado em seres humanos e foi primeiramente identificado através do isolamento de granulos de neutrólilos. hCAP-18 é produzida também por células epiteliais do pulmão, intestino, cavidade bucal e trato urogenital, sendo encontrada no plasma seminal e plasma sanguíneo. Após a secreção, ocorre a quebra de hCAP-18 pela ação de proteases em um peptídeo de cadeia longa LL-37, além de outros peptídeos menores. LL-37 é peptídeo catiônico com 37 resíduos, componente α-helicoidal do hCAP-18 (Low et al., 1999). É um modulador do sistema imuno inato, envolvendo funções como antividade antibacteriana, incluindo cepas bucais do grupo estreptococos (*S. mutans, S. sobrinus, S. mitis* e outras) (Ouhara et al., 2005). A função antibacteriana do LL-37 tem sido atribuída a sua capacidade de formar poros na membrana, além de, em altas concentrações, é citotóxica para células eucariotas (de Yang et al., 2000; Scott et al., 2002; Yang et al., 2004).

Os PCAM na saliva podem contribuir para a manutenção da saúde bucal geral e ter um papel junto à primeira linha do organismo contra as infecções (Mccormick e Weinberg, 2010; Wiesner e Vilcinskas, 2010; Zhang; Cherryholmes e Shively, 2008) uma vez que têem sido considerados contribuidores na saúde da mucosa oral, o que, provavelmente pode ser um fator biológico que influencia a suscetibilidade cariogênica (Dale et al., 2006). Alguns estudos relacionaram a presença de PCAM e CPI (Tao et al., 2005; Davidopoulou et al., 2012; Colombo et al., 2016). Tao et al*.,* 2005 observaram que baixos níveis de HNP1-3 podem representar um fator biológico para a susceptibilidade à cárie, já que foram detectadas maiores concentrações de HNP 1-3 em crianças livres de cárie. Davidopoulou et al. (2012) avaliaram os níveis de LL-37 em crianças com dentição decídua, mista e permanente com ou sem cáries e gengivite, verificando que crianças com dentição decídua mostraram concentrações significantemente menores do peptídeo, em relação às de dentição mista e permanente. O mesmo foi verificado para crianças com alta atividade de cárie quando comparadas às crianças com baixa ou moderada atividade de cárie. Em relação à ação

antimicrobiana desses peptídeos sobre microrganismos cariogênicos, estudo verificou que cepas de *S. mutans* isoladas de crianças com cárie ativa mostraram maior resistência a HNP-1-2, HBD-2-3 e LL-37 em concentrações variadas quando comparadas às crianças livres de cárie, demonstrando que essas cepas apresentam uma vantagem ecológica para a colonização mais efetiva do biofilme, aumentando o risco à doença cárie (Phattarataratip et al., 2011). Colombo et al. (2016) encontraram correlações positivas entre hBD-2, hBD-3 e LL-37, e entre esses, LL-37 foi o mais associado aos níveis de cárie. Assim, os PCAM poderiam ser uma alternativa aos antimicrobianos convencionais por serem alvos seletivos de células procariotas, diminundo a probabilidade de resistência microbiana (Zasloff, 2009).

Embora a etiologia da doença cárie seja multifatorial, a maioria dos seus fatores causadores é controlável, como a dieta, hábitos de higiene bucal, entre outros. Entretanto, a suscetibilidade do hospedeiro ao desenvolvimento da doença, representada pelas respostas imaturas ou deficientes do sistema imune, principalmente as crianças, poderia ser contornada utilizando-se PCAM como medida terapêutica direta para a redução da microbiota sem causar resistência antibiótica, por seu caráter natural ou ainda como medida indireta, modulando a resposta imunológica, favorecendo o melhor desempenho do organismo contra os patógenos. Assim, o objetivo deste estudo foi avaliar a citotoxicidade e atividade antimicrobiana em condições planctônicas e de biofilme de fragmentos derivados de peptídeos catiônicos (PC): LL-37 (originário de hCAP-18), D6-17 e D1-23 (originários de ortólogo da β-defensina-3 humana) contra bactérias cariogênicas.

Artigo

Cytotoxicity and effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions

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Abstract

This study evaluated the cytotoxicity and effect of fragments derived from oral cationic peptides (CP): LL-37, D6-17 and D1-23 against cariogenic bacteria under planktonic and biofilm conditions. For cytotoxicity analysis, two lines of epithelial cells were used. Minimum inhibitory concentration and minimal bactericidal concentration were determined for the CP fragments and control (chlorhexidine-CHX) against cariogenic bacteria. Fractional inhibitory concentration was obtained for the combinations of CP fragments on *Streptococcus mutans.* Biofilm assays were conducted with the best antimicrobial CP fragment against *S. mutans*. The results indicated that D6-17 was not cytotoxic. D1-23, LL-37 and CHX were not cytotoxic in low concentrations. D1-23 presented the best bactericidal activity against *S. mutans, S. mitis* and *S. salivarius.* Combinations of CP fragments did not show synergic effect. D1-23 presented higher activity against *S. mutans* biofilm than CHX. It was concluded that D1-23 showed a remarkable effect against cariogenic bacteria and low cytotoxicity.

Keywords: Dental Caries; Peptides; Cell Culture; Biofilms.

1. Introduction

Early childhood caries (ECC) represents the most common chronic disease in childhood with a prevalence of around 26% in Brazil (SB Brasil 2010) and 23% in the USA (Dye et al. 2015) among 5-6 year old children, and can be observed in toddlers as young as 12 months of age (SB Brasil 2010; Dye et al. 2015). ECC can progress and lead to severe destruction of primary teeth, causing infection, pain, chewing and speech difficulties, physiological trauma and early dental loss (Losso et al. 2009). Besides the negative effects on health, quality of life and high treatment costs, children who present ECC remain at a high risk for future caries recurrences and under continuous dental interventions, such as topical fluoride/antimicrobial applications (O'Sullivan and Tinanoff 1996).

By virtue of rapid tooth destruction in a short period of time, the association between the following factors has been suggested in the etiology of ECC: frequent intake of a diet rich in fermentable carbohydrates such as sucrose, high microorganism count and immunological vulnerability (Mattos-Graner et al. 1998, 2001). The bacterial group considered most cariogenic is mutans streptococci, especially *Streptococcus mutans*, one of the primary bacterial colonizers of dental enamel and less frequently *Streptococcus sobrinus* (Mattos-Graner et al. 2001, 2014; van Houte et al. 1991). However, other acidogenic and aciduric species, such as *Lactobacillus* and *Actinomyces*, are involved in the initiation of carious lesions (Sansone et al. 1993; van Houte et al. 1996).

The immune system has several forms of defense against pathogenic microorganisms. In addition to the physical barrier against the entrance of foreign microbes, mucous membranes are a source of potent antimicrobial cationic peptides (AMCP) (Mccormick and Weinberg 2010). They have an inhibitory action against a broad range of bacteria, fungi and enveloped viruses and promote modulation of the host immune response while maintaining normal microbiota in different niches such as the skin, intestine and oral cavity (Mccormick and Weinberg 2010; Wiesner and Vilcinskas 2010). The main AMCP found in saliva and/or crevicular fluid are α- and β-defensins (hBD) and cathelicidins, produced by epithelial cells (Abiko et al. 2003). Studies are focusing on the effect of hBD-3 against important oral pathogens, such as

S. mutans, S. sobrinus, Fusobacterium nucleatum and *Porphyromonas gingivalis* (Ouhara et al. 2005). The human cationic peptide (hCAP-18) is the only cathelicidin identified in humans, produced by epithelial cells from the lungs, gut, urogenital tract and oral cavity. After secretion, hCAP-18 is broken, by protease activity, to a small peptide called LL-37. This peptide fragment is a multifunctional immune modulator with antibacterial function and the ability to stimulate angiogenesis, skin healing and chemotaxis of inflammatory cells (Mccormick and Weinberg 2010; Wiesner and Vilcinskas 2010).

Limited research has been conducted to determine the effectiveness of synthetic or natural chemotherapeutic agents, individually or in combination, to prevent or reduce the incidence of ECC (Horowitz 1998). There has been recent interest in the use of peptides for the prevention of dental caries (Bernegossi et al. 2015; da Silva et al. 2013). Although AMCP have been pointed out as a new class of antibiotics, the long length of their amino acid chain or chemical linkages make their production as a therapeutic agent difficult. These peptides could serve as a template for the design of effective antibiotics for oral application, potentially reducing the cost of production and optimizing their antimicrobial properties (Batoni et al. 2011). Synthetic analogues of AMCP have reached clinical trials to be indicated for patients, as reported for defensin mimetic PMX-30063 and histatin-5 P113 (Gordon et al. 2005). The aim of this study was to evaluate the cytotoxicity and the effect of fragments derived from oral cationic peptides (CP): LL-37 (from hCAP-18), D6-17 and D1-23 (from ortologue of β-defensin-3) against cariogenic bacteria under planktonic and biofilm conditions.

2. Materials and methods

2.1 Preparation of peptides and controls

The peptide fragments LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) derived from hCAP-18 (Ji et al. 2007); Def14-1C^{V} (6-17) or D6-17 (LRKFFARIRGGR) and Defb14-1C^{V} (1-23) or D1-23 (FLPKTLRKFFARIRGGRAAVLNA) derived from Defb14, the mouse orthologue of human β-defensin-3 (Reynolds et al. 2010) were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Defb14-1C^{v} is the peptide with cysteines replaced with alanines except Cys_{40}, which resides at position V of the six-

cysteine motif. The synthetic peptides were resuspended in sterile deionized water at 20 mM and stored at -20˚C prior to their use. Chlorhexidine digluconate (CHX, Sigma Aldrich, St. Louis, MO, USA) was used as a control. All subsequent experiments were performed in triplicate, in three independent assays.

2.2 Cytotoxicity tests (Apêndice A)

2.2.1 Epithelial cell cultures

The following cell lines were tested: immortalized human gingival epithelial cell line OBA-9 and skin epidermal HaCaT. The OBA-9 cells were cultured in K-SFM serum-free medium (Life Technologies), containing insulin, epidermal growth factor, fibroblast growth factor and 100 µg mL^{-1} of penicillin G/streptomycin. The HaCaT cells were cultured in Dulbecco's modified Eagle's medium – DMEM (Gibco BRL, Carlsbad, CA, USA) plus 10% fetal calf serum and 100µg mL^{-1} penicillin G/streptomycin. Both cell lines were grown until they reached subconfluent density at 37^{o}C in 5% CO_2 (Bedran et al. 2014).

2.2.2 Stimulation of epithelial cells by peptide fragments

The epithelial cells were harvested following a trypsin treatment (5 min) (TrypLETM Express; Life Technologies Inc.) at 37°C. Proteases were then inactivated by adding 0.3 mg mL^{-1} of trypsin inhibitor and cells were harvested by centrifugation (500xg for 5 min), suspended in fresh medium, seeded in a 96-well microplate (200 µL/well, $1x10^6$ cells/mL) and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion before stimulation. The cells were then stimulated with the peptide fragments (LL-37; D6-17; D1-23) and CHX at concentrations ranging from 1 to 0.001mM for 24 h at 37°C in 5% CO_2 (Bedran et al. 2014).

2.2.3 Determination of cell viability

A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany), using 3-[4,5-diethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide as the substrate, was used to determine the effect of cationic peptide fragments and controls on cell viability. After exposure to the peptides and controls, the extracts were aspirated and replaced by 90 µL of DMEM or K-SFM plus 10 µL of MTT solution (5 mg mL^{-1} sterile PBS; Sigma

Aldrich). Next, the culture medium with the MTT solution was aspirated and replaced with 100 µL of acidified isopropanol solution. Two 50 µL aliquots of each well were transferred to 96-well plates. Cell viability was evaluated using spectrophotometry, being proportional to the absorbance measured at 570 nm wavelengths with an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The means were calculated for the groups and transformed into percentages, which represented the inhibitory effect of the mitochondrial activity of the cells by peptides/CHX. The negative control (DMEM or K-SFM) was defined as having 100% cell metabolism (Bedran et al. 2014).

2.2.4. DAPI staining

Apoptotic nuclear morphology was observed using 4,6-Diamidino-2-phenylindole dihydrochoride (DAPI). HaCaT cells at a density of 2x 10^5 cells/well were placed onto 24-well slides and treated with 0.1mM of each peptide fragment or CHX for 24h. Next, cells were washed with phosphate-buffered saline (PBS) and stained with DAPI solution, as described previously (Lai et al. 2011). After staining, the cells were examined and photographed using a fluorescence microscope (Leica, DM5500 B, Wetzer, Hesse, Germany).

2.3 Antimicrobial tests

2.3.1 Bacterial conditions

The following bacterial strains used in the present study were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil): *Streptococcus mutans* (ATCC – 25175), *Streptococcus mitis* (ATCC 4945), *Streptococcus oralis* (IAL - 1676), *Streptococcus sanguinis* (ATCC 10557), *Streptococcus salivarius* (ATCC 7073), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus paracasei* (ATCC 335), *Lactobacillus rhamnosus* (ATCC 9595), *Lactobacillus brevis* (ATCC 367), *Lactobacillus fermentum* (ATCC 9338) and *Actinomyces israelii* (ATCC 12102). Clinical *S. mutans strains* 1 and 2 (CS1 and CS2) were kindly provided by Dr. Renata Mattos-Graner and were previously isolated from ECC children and characterized as highly cariogenic (Mattos-Graner et al. 2004). The purity of the strains was confirmed using the Gram's method. Microbial suspensions were prepared from culture previously grown in Mitis

Salivarius Agar Base (Difco Laboratories, Detroit, MI, USA) with 0.2 U mg mL^{-1} Bacitracin (Sigma-Aldrich) for *S. mutans* strains, Mitis Salivarius Agar (Difco Laboratories) for the other *Streptococcus* strains (Difco Laboratories), Rogosa Agar (Difco Laboratories) for *Lactobacillus* and Brain Heart Infusion Agar (Difco Laboratories) for *Actinomyces* and incubated at 37°C for 24 h in 5% CO_2. Growth curve assays were performed for each bacterium in order to determine the optical density (OD) at the mid-log phase with approximately 5-10x10^{8} CFU mL^{-1} to be used in the following experiments. The absorbance was measured using a microplate reader (Eon Microplate Spectrophotometer, BioTek Instruments, Winooski, VT, USA) to assess cell density.

2.3.2 Determination of MIC and MBC (Apêndice B)

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution method, in 96-well microtiter plates, following the criteria previously described by the Clinical Laboratory Standards Institute M7-A9 (CLSI 2012) for bacteria. Bacterial cell cultures at the mid-log phase were harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at 3000xg, the supernatant was discarded and the pellet re-suspended in Mueller-Hinton broth (Difco Laboratories). The final concentration of bacterial suspension in the wells was 5-10x10^{5} CFU mL^{-1}. The fragments of cationic peptides were serially diluted in sterile deionized water at concentrations ranging from 1 to 0.001 mM. Next, bacterial suspension was inoculated in each well. The plates were incubated at 37°C for 24 h in 5% CO_2. Afterwards, 15 µL of 0.01% resazurin staining (Sigma-Aldrich) was applied to each well and incubated for 4 h to determine cell viability (Hahnel et al. 2012). After that, wells corresponding to MIC and at least three previous wells were homogenized, serially diluted and plated on Mueller-Hinton agar to determine the MBC. The plates were incubated at 37°C for 24 h in 5% CO_2. The number of colonies forming units/mL (CFU mL^{-1}) of bacteria was determined. The MBC was considered when the peptides killed 99.9% of the tested bacterial culture. CHX and the culture medium were used as positive and negative controls, respectively.

2.3.3 Determination of FIC

The combined effects of peptide fragments were evaluated by the fractional inhibitory concentration (FIC) index, using the checkerboard assays as previously described (Tong et al. 2011). Briefly, the rows of a 96-well microplate contained the same concentrations of one of the peptide fragments (or two peptides for triple combinations), diluted from 1 to 0.001 mM along the y-axis. The column contained the same concentration of another peptide fragment, diluted from 1 to 0.001 mM along the x-axis. After 24h of incubation at 37°C in 5% CO_2, plates were stained with resazurin for 4 h. The FIC index was calculated according to the equation: FIC index = FIC A (MIC of antimicrobial A in combination/MIC of A alone)+ FIC B (MIC of antimicrobial B in combination/MIC of B alone). The FIC values were interpreted as synergy if the values were ≤0.5, no interaction if the values were between 0.5 and 4.0 and antagonism if the values were >4.0.

2.3.4. Biofilm assays (Apêndice C)

Biofilm assays were conducted with the peptide fragment, which demonstrated the best bactericidal effect against *S. mutans* strains (D1-23). This part of the study was reviewed and approved by the Animal and Human Research Ethics Committee of Araçatuba Dental School, Universidade Estadual Paulista, Brazil (Protocols:198/2013 and #CAAE 13079213.4.0000.5420) (Anexo A). These assays were based on the study of Ccahuan-Vásquez and Cury (2010) with some modifications. Enamel blocks (2 mmx2mmx2 mm) from bovine incisor teeth were cut and sequentially polished and selected through measurement of surface free energy (Drop Shape Analyzer – DSA100, Krüss, GmbH, Hamburg, Germany)(Brambilla et al. 2012). The mean±standard deviation of free energy on the enamel blocks was 114±15 mM/m and they were carefully randomized and distributed into three groups (n=6): negative control (culture medium), D1-23 and CHX. The enamel blocks had been previously sterilized in water inside glass tubes at 121°C for 30 min and their sterility was tested before use (Amechi et al. 1998) (Apêndice D). The enamel blocks were fixed with double sided tape to the bottom of sterile polystyrene 96-well microplates, with a U-shaped base, and pretreated with 200 µL of the stimulated saliva per well for 4 h at 37°C in 5% CO_2 (coating phase). The saliva was previously centrifuged at 3000xg for 10 min and the

supernatant filtered through a 0.22 µm membrane filter (Corning Inc., Corning, USA). After the incubation time, the saliva was removed and 10 µL of each microorganism suspension (approximately 5-10x10^6 CFU mL^{-1}) was inoculated in each well containing 90 µL of BHI broth supplemented with 1% sucrose. The plates were incubated at 37° in a 5% CO_2 atmosphere. After 48 h, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 200 µL of D1-23, CHX and water. The concentrations used for these assays were 2 and 10 times higher than the MBC concentration. The microplates were incubated in the same conditions for 24 h. Specimens were carefully removed from the wells, washed in saline and individually transferred to microtubes containing 1mL of saline and sonicated at 7W for 30 s (Branson, Sonifier 50, Danbury, CT, USA) to detach cells from the biofilm formed on the enamel specimens (Ccahuana-Vásquez and Cury 2010). Aliquots of the suspension were diluted and inoculated in BHI Agar (Difco Laboratories). The plates were incubated for 48 h at 37° C, 5% CO_2. After this period, bacterial colonies were counted and expressed in CFU mL^{-1}.

2.3.5. Confocal Laser Scanning Microscopy (CLSM) (Apêndice E)

Biofilm assays for CLSM analysis were conducted with *S. mutans* ATCC testing the peptide that showed the best antimicrobial activity on MIC/MLC assays. Enamel blocks (n = 6) measuring 3mmx3mmx0.5 mm were inserted in wells, as methodology described above and biofilm was formed during 48h and exposed to peptide (D1-23) or CHX at 10X MBC concentration for 24h. After this period, enamel blocks were washed once with sterile deionized water and stained with 100 µL of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide, according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Two additional uninfected specimens were stained under the same protocol and used as negative control. Fluorescence from the stained cells was viewed by CLSM (Leica TCS SP5, Leica Microsystems, Lincolnshire, IL, USA), using 63x oil immersion lens. CLSM images were acquired using software (LAS AF Leica Microsystems) at a resolution of 1024 by 1024 pixels. Ten-micrometer-deep scans (0.2-µm step size) were obtained from two randomly selected places with the CLSM. In

order to analyze the Live/Dead cells ratios on enamel slices, all scans were reconstructed in a three-dimensional model by the same software. The quantification of red fluorescence ratio in relation to green-and-red fluorescence and biofilm thickness were determined by software Image J 1.48 (NIH, Bethesda, MA, USA) (Lee et al. 2013).

2.4 Statistical analysis

Data from cytotoxicity were submitted to the ANOVA/Tukey tests in order to compare the effects of the peptide fragments on epithelial cells, considering each concentration separately. Box-whisker plots were performed to represent the distribution of non-parametric data obtained in the biofilm assays and Mann-Whitney tests were applied to compare D1-23 with CHX for each *S. mutans* strain. CLSM data (quantification of dead cells in relation to total cells and biofilm thickness) were converted in means/standard deviations and submitted to ANOVA and Tukey tests ($p<0.05$). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis.

3. Results

3.1 Cytotoxicity tests

Figures 1 and 2 present the percentage of the HaCat and OBA-9 metabolisms, respectively. After 24h of exposure, D6-17 did not affect the cell metabolism of either epithelial line in the concentrations tested. D1-23 showed toxicity at concentrations higher than 0.2mM for both epithelial cells. LL-37 and CHX were the most cytotoxic peptides, demonstrating toxicity in concentrations above 0.02 mM for both cells. Figure 3 shows representative fluorescence microscopic images of HaCaT cells exposed to peptide fragments at 0.1mM. A higher intensity DAPI-staining cells and the presence of fragmented nuclei, as a consequent of nuclear condensation and chromatin shrinkage, were observed for LL-37 group and cells with the aspect of apoptosis in the CHX group. Cells treated with D1-23 and D6-17 presented morphology similar to the control group (culture medium).

3.2 Antimicrobial activity (in planktonic conditions)

Table 1 shows the MIC and MBC values obtained for the cationic peptide fragments. CHX demonstrated the best antibacterial effect against all bacteria tested. Among the peptide fragments, D1-23 presented the best bactericidal activity against *S. mutans* strains*, S. mitis* and *S. salivarius* with MIC values ranging from 0.003 mM to 0.1 mM and MBC ranging from 0.005 to 0.2 mM. D1-23 did not have an effect on *S. oralis* or *S. sanguinis*, however it presented good results against *Lactobacillus* spp. and *Actinomyces israelii* (MIC/MBC range: 0.0003-0.4 mM). LL-37 demonstrated a superior effect against the *Lactobacillus* and *Actinomyces* species tested; however, its action on *Streptococcus* spp. was lower when compared to D1-23. D6-17 showed bactericidal activity only against *S. mutans* strains*, L. brevis and L. fermentum.* Table 2 presents the FIC values for peptides combinations and no synergic effect was observed against *S. mutans*.

3.3 Effect against S. mutans biofilm

Box-whisker plots showed a reduction in the percentage of *S. mutans* after 24h of D1-23 and CHX exposure. Both agents improved their activity against biofilm of all *S. mutans* strains with an increase in concentration (2 to 10x MBC). At 2x MBC, CHX presented a similar effect for all strains tested. D1-23 at 2x MBC was superior to CHX only against *S. mutans* ATCC. This strain was more sensitive to D1-23 than the other *S. mutans* strains, showing the highest bacterial reduction at 10X MBC. D1-23 demonstrated a better effect against *S. mutans* biofilm than CHX at 10x MBC, except for *S. mutans* CS1 (Figure 4). Representative images obtained from CLSM analysis from *S. mutans* biofilm on enamel blocks are observed in Figure 5A. D1-23 and CHX showed higher quantification of dead cells (red points) when compared to control (culture medium) which presented strong predominance of live cells (green points). D1-23 presented higher activity against biofilm of *S. mutans* than CHX and control groups*,* with an average of 71% of cell death (Figure 5B). The biofilm thickness were lower in the D1-23 group compared to CHX and control groups, as shows in Figure 6A and 6B.

4. Discussion

The aim of this study was to identify smaller sequences with the same or better antimicrobial activity as the original peptides without cytotoxicity, reducing costs and difficulties with their synthesis and other limitations. In the present study, we evaluated three cationic peptides-derived fragments D6-17, D1-23 and LL37 with positive charges of 5, 6 and 6, pH 7.0, respectively. Reynolds et al. (2010) synthesized several overlapping fragments of Defb14 (mouse orthologue of human β-defensin 3) and Defb14-1C^{v} (peptide with cysteines replaced with alanines except Cys_{40}, which resides at position V of the six-cysteine motif) and determined planktonic antimicrobial activity. Defb14-1C^{v} (D1-23) and Defb14-1C^{v} (D6-17) had the best MBCs against Gram-positive and Gram-negative bacterial strains. LL-37 was also chosen for this study based on antimicrobial activity reported by previous studies (Gordon et al. 2005; Ouhara et al. 2005).

One limitation to the use of peptides as future drugs is the toxicity of peptides to the host cell at therapeutic concentrations. In our study, D6-17 did not affect cell metabolism and D1-23 presented good results up to 0.2mM for both cell lines. It is well-known that cationic peptides not only interact with microorganisms, but also with eukaryotic cells, causing toxicity. Hydrophobic interactions occur between anionic phosphatidylglycerol from bacterial cell membranes and with zwitterionic phosphatidylcholine and phosphatidylethanolamine from mammalian cells (Yeaman and Yount 2003). Cytotoxicity of hBD-3 fragments are related to hydrophobicity, which decreases as the length of the peptide decreases (Reynolds et al. 2010). D1-23 was more cytotoxic than D6-17 as it possesses a longer N-terminal length and consequently higher hydrophobicity (Ji et al. 2007). Our results are in agreement with Kluver et al. (2005), which tested the cytotoxicity of hBD-3 peptides with three disulfide bonds and the analogues containing alanine and tryptophan for the cysteine residues. They showed that replacement of cysteine residues with alanine caused a reduction in the overall hydrophobicity, making the analogues less cytotoxic. Alanine has one methyl group in its side chain while cysteine has a sulfhydryl. D1-23 and D6-17 are alanine-substituted hBD-3 variants demonstrating an advantage in relation to disulfide bonds in peptides or analogues containing tryptophan, which exhibit a markedly higher potency in reducing human monocytic cell viability (Yeaman and

Yount 2003). In contrast, Liu et al. (2008) also tested the cytotoxicity of linear analogues of hBD3 and regardless of their hydrophobicity; they showed reduced epithelial toxicity when compared with wild-type hBD3 in the concentration range of 6.25–200 μg mL^{-1}.

In the present study, LL-37 was the most toxic peptide fragment to epithelial cells, reducing its cytotoxicity in concentrations below 0.02mM. DAPI fluorescence analysis confirmed the toxic effect of LL-37 at 1mM causing fragmentation of cell nuclei. The effect of LL-37 on mammalian cells, but not epithelial cells, was first studied by Johansson et al. (1998) who observed cytotoxicity at 13–25μM, which gradually increased at higher concentrations. In a culture of gingival epithelial cells (HGEC), similar to OBA-9 lines, obtained from gingival tissue overlying impacted third molars of patients, doses of LL-37 up to 6 μM did not significantly decrease the percentage of HGEC survival (Montreekachon et al. 2014). In the human body, a high concentration of LL-37 is controlled by its binding to plasma proteins, such as apolipoprotein A-I, reducing both cytotoxicity and antimicrobial activity (Ciornei et al. 2005). Studies have focused on more active fragments or analogues of LL-37 with a less cytotoxic effect (Ciornei et al. 2005; Johansson et al. 1998). All peptide fragments were less cytotoxic than CHX solution. DAPI analysis showed aspect of apoptosis in epithelial cells treated with CHX, as observed in different types of cells in another studies (Gianelli et al. 2008; Rocha et al. 2014). CHX has been pointed as an apoptosis-promoting agent because it induces disturbance of mitochondrial function, intracellular Ca^{+2} increase and oxidative stress (Gianelli et al. 2008).

For the present study, some early (*S. sanguinis, S. mitis* and *S. oralis*. *Actinomyces* spp.) and late (*S. mutans, S. sobrinus, Lactobacillus* spp.) bacterial species related to dental biofilm formation were chosen to test the antimicrobial activity of cationic peptide fragments. Among the fragments of peptides, D1-23 demonstrated the best bactericidal activity against *S. mutans* strains*, S. mitis* and *S. salivarius* and good results against *Lactobacillus* spp. and *Actinomyces israelii*. Reynolds et al. (2010) discovered that the 23-amino-acid N-terminal half of Defb14-$1C^{V}$ is a potent antimicrobial region while the C-terminal half is not. The regions containing the amino acids 1 to 10 and 6 to 17 demonstrated a potent bactericidal effect; however D6-17 showed a strong inhibitory action against Gram-negative (Reynolds et al. 2010). In the

present study, D1-23 was more effective than D6-17 against cariogenic bacteria, confirming the better action of D1-23 on Gram-positive bacteria compared to other Defb14 peptide fragments (Reynolds et al. 2010). Biofilm reduction was observed for D1-23 at 10x MBC against *S. mutans* strains, superior to CHX solution, except for *S. mutans* CS1. Confocal analysis also showed higher effect against *S. mutans* biofilms and thinner biofilm for D1-23 when compared to CHX and control groups. The effect of antimicrobial agents on biofilms depends on several factors, such as depletion in the fluid phase and penetration of antibiotics and physiology (stages of growth) of biofilms. The first and last factors are probably not general causes of biofilm tolerance *in vitro* models. However, the penetration times could interfere in the ability of antibiotics reduces the biomass of biofilms. Conversely as the intuition suggests, penetration times do not increase with the molecular weight of the antimicrobial agent. Even large antibiotics and antimicrobial peptides can penetrate a biofilm within a few minutes. Some examples of large agents that penetrate rapidly within biofilms are vancomycin (0.5 min), daptomycin (1.5 min), and nisin (4 -10 min) (Stewart et al. 2015)

No study was found evaluating the effect of D1-23 and D6-17 against biofilms, however, their human original form, hBD-3, has exhibited more antibacterial activity against mature multispecies biofilms with *S. mutans, A. naeslundii, L. salivarius* and *E. faecalis* than CHX (Lee et al. 2013). Both structure and sequence are important for the antimicrobial activity of these β-defensin derivatives (Reynolds et al. 2010). The mechanism of action of defensins is associated with peptide binding to the bacterial cell membrane. Ionic interaction of cationic defensins with negatively charged phospholipids causing permeabilization and cell lysis (Abiko et al. 2003; Ganz 2003). Sahl et al. (2005) revised the mechanism of antibiotic activity of mammalian defensins and found that membrane depolarization contributes to rapid killing of a significant number of bacterial cells within a culture. However, subpopulations appear to survive and growth or be killing through additional activities of the peptides, such as the activation of cell-wall lytic enzymes.

Another important cationic peptide tested in the present study was LL-37. This peptide demonstrated a superior effect against the *Lactobacillus* and *Actinomyces* species tested; however, its action on *Streptococcus* spp. was lower when compared to

D1-23. LL-37 acts on the outer membrane of bacterial cells binding with the positively charged amino acids in contact with the head groups of the phospholipids. The accumulation of peptides causes small toroidal pores that lead to severe leakage. Additionally, the inner membrane is covered in a carpet-like manner and perturbated, becoming intracellular targets such as DNA susceptible to binding with LL-37. Electrostatic interaction with protein complexes responsible for electron transport may also occurred with LL-37, generating ATP, which could lead to the disruption of membrane homeostasis (reviewed by Vandamme et al 2012). Ouhara et al. (2005) evaluated the inhibitory effect of LL-37 on the following cariogenic bacteria: *S. mutans, S. sobrinus, S. salivarius, S. sanguinis, S. mitis* and *L. casei* and found MIC ranging from 25-50 μg mL^{-1}(around 0.01mM), lower than obtained by the present study. In contrast to the present study, the authors found a superior effect of LL-37 against *streptococci* when compared to *L. casei.* In the present study, synergism was not observed among peptide fragments. LL-37 and hBD-3 had a synergic effect on killing *S. aureus* at pH 8.0 and 7.4 that was eliminated at pH 6.8 (Abou et al., 2014). *S. mutans* is considered highly acidogenic (Mattos-Graner et al. 2014; van Houte et al. 1991) and the pH of the culture medium could have interfered in the synergism of peptides.

There is a demand for novel antimicrobials due to the current trend of a reduction in the potency of commonly used antibiotics and peptides could be an alternative to conventional antimicrobials, because they selectively target prokaryotes and minimally trigger the emergence of microbial resistance. However, native peptides tend to be easily degraded, are expensive to produce and have been shown to be toxic in their active forms (Abiko et al. 2003; Batoni et al. 2011; Wiesner and Vilcinskas 2010). The design of synthetic fragments of peptides with a broad-spread action against bacterial pathogens, low toxicity to the host and low production cost could be interesting for oral application as a preventive method for caries prevention.

5. Conclusion

Considering the limited number of peptide fragments tested in the present study, D1-23 presented a remarkable effect against cariogenic bacteria under planktonic and biofilm conditions and low toxicity to epithelial cells.

Disclosure statement

The authors declare that they have no conflict of interest.

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References

Abiko Y, Nishimura M, Kaku T. 2003. Defensins in saliva and the salivary glands. Med Electron Micros. 36:247-252.

Abou Alaiwa MH, Reznikov LR, Gansemer ND, Sheets KA, Horswill AR, Stoltz DA, Zabner J, Welsh MJ. 2014. pH modulates the activity and synergism of the airway surface liquid antimicrobials b-defensin-3 and LL-37. Proc Natl Acad Sci U USA. 111:18703:18708.

Amechi BT, Higham SM, Edgar WM. 1998. Efficacy of sterilization methods and their effect on enamel demineralization. Caries Res. 32:441-446.

Batoni G, Maisetta G, Brancatisano FL, Esin S, Campa M. 2011. Use of antimicrobial peptides against microbial biofilms: advantages and limits. Curr Med Chem. 18:256-279.

Bedran TB, Mayer MP, Spolidorio DP, Grenier D. 2014. Synergistic anti-inflammatory activity of the antimicrobial peptides human beta-defensin-3 (hBD-3) and cathelicidin (LL-37) in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. PLoS One. 9:e106766.

Benergossi J, Calixto G, Fonseca-Santos B, Aida KL, Negrini TC, Duque C, Gremião MP, Chorilli M. 2015. Highlights in peptide nanoparticle carriers intended to oral diseases. Curr Top Med Chem. 15:345-355.

Brambilla E, Ionescu A, Gagliani M, Cochis A, Arciola CR, Rimondini L. 2012. Biofilm formation on composite resin for dental restorations: an in situ study of the effect of chlorhexidine mouthrinses. Int J Artif Organs. 35:792-799.

Ccahuana-Vásquez RA, Cury JA. 2010. *S. mutans* biofilm model to evaluate antimicrobial substances and enamel demineralization. Braz Oral Res. 24:135-141.

Ciornei CD, Sigurdardóttir T, Schmidtchen A, Bodelsson M. 2005. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrob Agents Chemother. 49:2845-2850.

CLSI - Clinical and Laboratory Standard Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed., Wayne, PA, CLSI document, 2012, M7-A9.

da Silva BR, de Freitas VA, Carneiro VA, Arruda FV, Lorenzón EN, de Aguiar AS, Cilli EM, Cavada BS, Teixeira EH. 2013. Antimicrobial activity of the synthetic peptide Lys-a1 against oral streptococci. Peptides. 42:78-83.

Dye BA, Hsu KL, Afful J. 2015. Prevalence and Measurement of Dental Caries in Young Children. Pediatr Dent. 37:200-216.

Giannelli M, Chellini F, Margheri M, Tonelli P, Tani A. 2008. Effect of chlorhexidine digluconate on different cell types: a molecular and ultrastructural investigation. Toxicol In vitro 22:308-17.

Ganz T. 2003. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 3:710–720.

Gordon YJ, Romanowski EG, McDermott AM. 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. 30:505–515.

Hahnel S, Mühlbauer G, Hoffmann J, Ionescu A, Bürgers R, Rosentritt M, Handel G, Häberlein I. 2012. *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation and metabolic activity on dental materials. Acta Odontol Scand. 70:114-21.

Horowitz HS. 1998. Research issues in early childhood caries. Community Dent Oral Epidemiol. 26:67–81.

Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y. 2007. Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. J Periodontal Res. 42:410-419.

Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B. 1998. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J Biol Chem. 273:3718-3724.

Klüver E, Schulz-Maronde S, Scheid S, Meyer B, Forssmann WG, Adermann K. 2005. Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. Biochemistry. 44:9804-9816.

Lai WW, Hsiao YP, Chung JG, Wei YH, Cheng YW, Yang JH. 2011. Synergistic phototoxic effects of glycolic acid in a human keratinocyte cell line (HaCaT). J Dermatol Sci. 64: 191-198.

Lee JK, Chang SW, Perinpanayagam H, Lim SM, Park YJ, Han SH, Baek SH, Zhu Q, Bae KS, Kum KY. 2013. Antibacterial efficacy of a human β-Defensin-3 peptide on multispecies biofilms. J Endod. 39:1625-9

Liu S, Zhou L, Li J, Suresh A, Verma C, Foo FH, Yap EP, Tan DT, Beuerman RW. 2008. Linear analogues of human beta-defensin 3: concepts for design of antimicrobial peptides with reduced cytotoxicity to mammalian cells. Chembiochem. 9:964-973.

Losso EM, Tavares MC, Silva JY, Urban CA. 2009. Severe early childhood caries: an integral approach, J Pediatr. 85:295-300.

Mattos-Graner RO, Correa MSNP, Latorre MRO, Peres RCR, Mayer MPA. 2001. Mutans streptococci oral colonization in 12-30-month-old Brazilian children over a one year follow-up period. J Public Health Dent. 61:161-167.

Mattos-Graner RO, Klein MI, Smith DJ. 2014. Lessons learned from clinical studies: roles of mutans streptococci in the pathogenesis of dental caries. Curr Oral Health Rep. 1:70–78.

Mattos-Graner RO, Napimoga MH, Fukushima K, Duncan MJ, Smith DJ. 2004. Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes. J Clin Microbiol. 42:4586-4592.

Mattos-Graner RO, Zelante F, Line RC, Mayer MP. 1998. Association between caries prevalence and clinical, microbiological and dietary variables in 1.0 to 2.5-year-old Brazilian children. Caries Res. 32:319-323.

Mccormick TS, Weinberg A. 2010. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. Periodontol 2000. 54:195–206.

Montreekachon P, Nongparn S, Sastraruji T, Khongkhunthian S, Chruewkamlow N, Kasinrerk W, Krisanaprakornkit S. 2014. Favorable interleukin-8 induction in human gingival epithelial cells by the antimicrobial peptide LL-37. Asian Pac J Allergy Immunol. 32:251-260.

O'Sullivan DM, Tinanoff N. 1996. The association of early childhood caries patterns with caries incidence in pre-school children. J Public Health Dent. 56:81-83.

Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M. 2005. Susceptibilities of periodontopathogenic and cariogenicbacteria to antibacterial peptides, {beta}-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother. 55:888–896.

Reynolds NL, De Cecco M, Taylor K, Stanton C, Kilanowski F, Kalapothakis J, Seo E, Uhrin D, Campopiano D, Govan J, Macmillan D, Barran P, Dorin JR. 2010. Peptide fragments of a beta-defensin derivative with potent bactericidal activity. Antimicrob Agents Chemother. 54:1922-1929.

Rocha RS, Meireles JR, de Moraes MCE. 2014. Chromosomal damage and apoptosis analysis in exfoliated oral epithelial cells from mouthwash and alcohol users. Genet Mol Biol. 37:702-7.

Sahl HG, Pag U, Bonness S, Wagner S, Antcheva N, Tossi A. 2005. Mammalian defensins: structures and mechanism of antibiotic activity. J Leukoc Biol.77(4):466-75.

Sansone C, van Houte J, Joshipura K, Kent R, Margolis HC. 1993. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res. 72:508-516.

SBBrasil. Brazilian Oral Health Report 2010. Available from: http://dab.saude.gov.br/CNSB/sbbrasil/arquivos/projeto_sb2010_relatorio_final.pdf. [Dec 30th, 2015].

Stewart PS. Antimicrobial tolerance in biofilms. 2015. Microbiol Spectr. 3(3) doi:10.1128/microbiolspec.MB-0010-2014.

Tong Z, Zhou L, Jiang W, Kuang R, Li J, Tao R, Ni L. 2011. An in vitro evaluation of the use of nisin and sodium fluoride or chlorhexidine against *Streptococcus mutans. Peptides*. 32:2021-6.

van Houte J, Lopman J, Kent R. 1996. The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J Dent Res. 75:1008-1014.

van Houte J, Sansone C, Joshipura K, Kent R. 1991. In vitro acidogenic potential of mutans streptococci of human smooth-surface plaque associated with initial caries lesions and sound enamel. J Dent Res. 7:1497-1502.

Vandamme D, Landuyt B, Luyten W, Schoofs L. 2012. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell Immunol. 280:22-35.

Wiesner J, Vilcinskas A. 2010. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 1:440-464.

Yeaman MR, Yount NY. 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacological Reviews. 55:27-55.

Tables and Figures

Table captions

Table 1. Values of MIC and (MBC)[†] in mM obtained by cationic peptide fragments against oral bacteria.
Note: † MIC – Minimum Inhibitory Concentration; MBC – Minimal Bactericidal Concentration

Table 2. Values of FIC obtained by combinations of fragments of cationic peptides against *S. mutans* (ATCC 25175).
Note:*FIC index = FIC A+FIC B = (MIC of antimicrobial A in combination/MIC of A alone) + (MIC of antimicrobial B in combination/MIC of B alone). Synergism was defined as an FIC index <0.5; no interaction as an FIC index of 0.5–4.0 and antagonism as an FIC index >4.0.

Figure captions

Figure 1. Mean (bars = standard deviation) of percentage of HaCat cell metabolism (MTT assay) after CHX and peptide fragments exposure.
Note: [a] Different lower letters show statistical difference among the groups, considering each concentration separately, according to ANOVA and Tukey tests.

Figure 2. Mean (bars = standard deviation) of percentage of OBA-9 cell metabolism (MTT assay) after CHX and peptide fragments exposure.
Note: [a] Different lower letters show statistical difference among the groups, considering each concentration separately, according to ANOVA and Tukey tests.

Figure 3. Fluorescence microscopic images (x200) of HaCaT cells treated with peptide fragments (LL-37, D1-23 and D6-17) and CHX for 24h. Red narrows shows higher intensity DAPI-staining cells and the presence of fragmented nuclei observed for LL-37 group. White narrows shows cells with aspect of apoptosis in the CHX group. Control = culture medium (DMEM).

Figure 4. Box-whisker plots of the activity of D1-23 (2X MBC and 10X MBC) and CHX (2xMBC and 10xMBC) against biofilm of *S. mutans* ATCC and clinical strains (CS1 and CS2). Different capital letters show statistical difference among the groups, considering each strain separately, according to Mann-Whitney tests.
Note: *Bars indicate minimum and maximum values. Boxes indicate lower and upper quartiles. Line in the middle of boxes is median.*

Figure 5. Confocal microscopy (CLSM) of *S. mutans* biofilms on enamel blocks after exposure to D1-23 and CHX. **A.** Representative images (x63 immersion) of dead cells and live cells and merge images of *S. mutans* biofilms. D1-23 and CHX showed higher quantification of dead cells (red points) when compared to control (no treatment) which presented strong predominance of live cells (green points). **B.** Means (bars-standard deviations) of percentage of dead cells obtained after CLSM analysis of *S. mutans* biofilm. Note: [a]Different lower case letter show statistical differences among the groups, according to ANOVA and Tukey tests ($p<0.05$).

Figure 6. Biofilm thickness. **A.** Representative 3D images obtained by CLSM of *S. mutans* biofilms after D1-23 and CHX exposure for 24h. **B.** Means (bars-standard deviations) of biofilm thickness obtained after CLSM analysis of *S. mutans* biofilm. Note: [a]Different lower case letter show statistical differences among the groups, according to ANOVA and Tukey tests ($p<0.05$). Control = no treatment

Table 1. Values of MIC and (MBC)[†] obtained by cationic peptide fragments against oral bacteria.

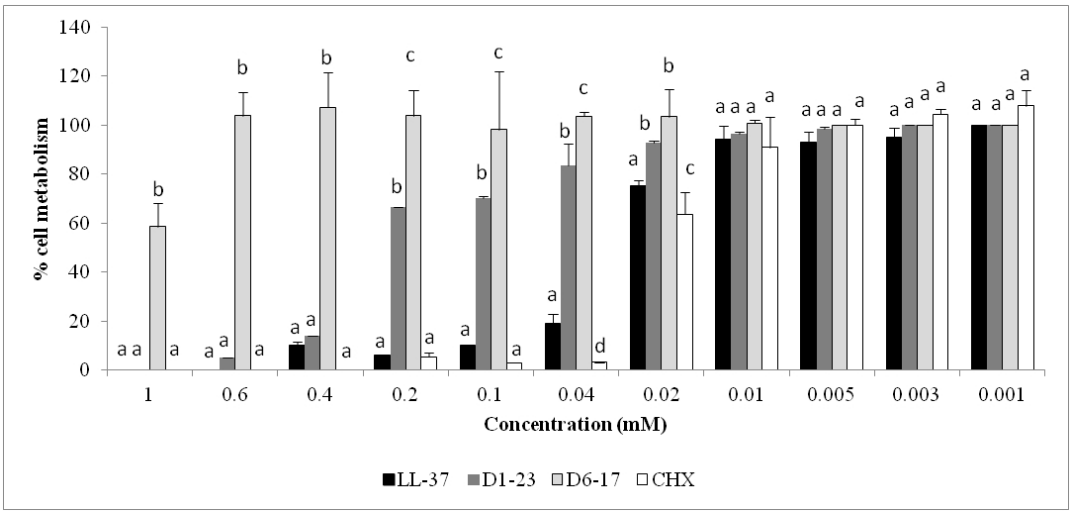
	LL-37 mM	D1-23 mM	D6-17 mM	CHX mM
S. mutans (*ATCC*)	0.1 (0.2)	0.01(0.02)	0.1 (0.2)	0.001 (0.003)
S. m clinical strain 1 (CS1)	0.2 (0.2)	0.003 (0.005)	0.4 (0.4)	0.001(0.001)
S. m clinical strain 2 (CS2)	0.1 (0.1)	0.01(0.01)	0.1(0.2)	0.001 (0.003)
S. salivarius	0.005(0.01)	0.005 (0.01)	0.4 (0.6)	0.001 (0.005)
S. mitis	0.01 (0.02)	0.1 (0.2)	0.6 (>1)	0.005(0.005)
S. oralis	0.2 (0.4)	>1(>1)	1(>1)	0.02 (0.02)
S. sanguinis	>1(>1)	>1(>1)	>1(>1)	0.01 (0.02)
L. acidophilus	0.01 (0.01)	0.4(0.4)	>1(>1)	0.003 (0.02)
L. casei	0.02(0.2)	0.4 (>1)	>1(>1)	0.003 (0.02)
L. rhamnosus	0.01 (0.01)	0.4 (0.4)	>1 (>1)	0.003 (0.003)
L. brevis	0.001 (0.001)	0.003 (0.003)	0.02(0.02)	0.001 (0.001)
L. fermentum	0.001(0.003)	0.005(0.02)	0.4 (0.6)	0.001(0.005)
Actinomyces israelii	0.005 (0.04)	0.02 (0.2)	0.4 (>1)	0.005 (0.02)

† MIC – Minimum Inhibitory Concentration; MBC – Minimal Bactericidal Concentration

Table 2. Values of FIC obtained by combinations of fragments of cationic peptides against *S. mutans* (ATCC 25175).

	FIC*	**Effect**
Double combination		
D6-17+D1-23	2.4	No interaction
D6-17+LL37	2.4	No interaction
D1-23+LL-37	2.1	No interaction
Triple combination		
D6-17+D1-23+LL-37	2.5	No interaction

*FIC index = FIC A+FIC B = (MIC of antimicrobial A in combination/MIC of A alone) + (MIC of antimicrobial B in combination/MIC of B alone). Synergism was defined as an FIC index #0.5; no interaction as an FIC index of 0.5–4.0 and antagonism as an FIC index >4.0.



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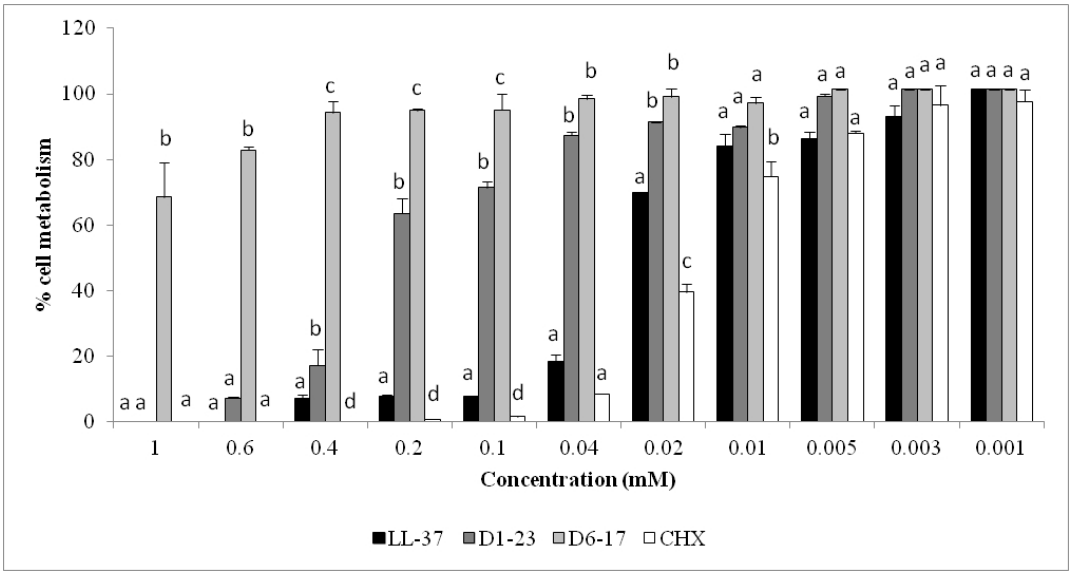

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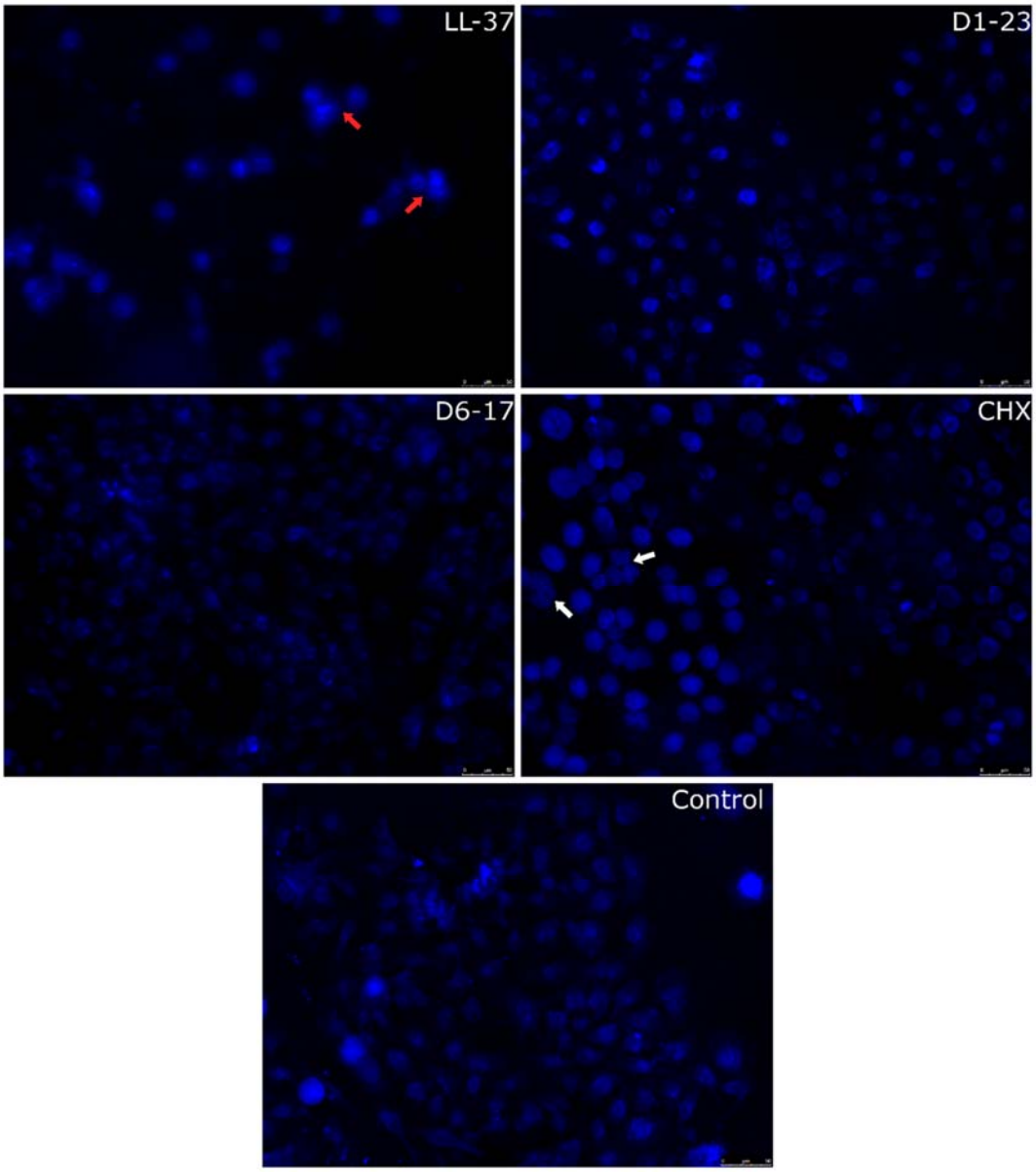

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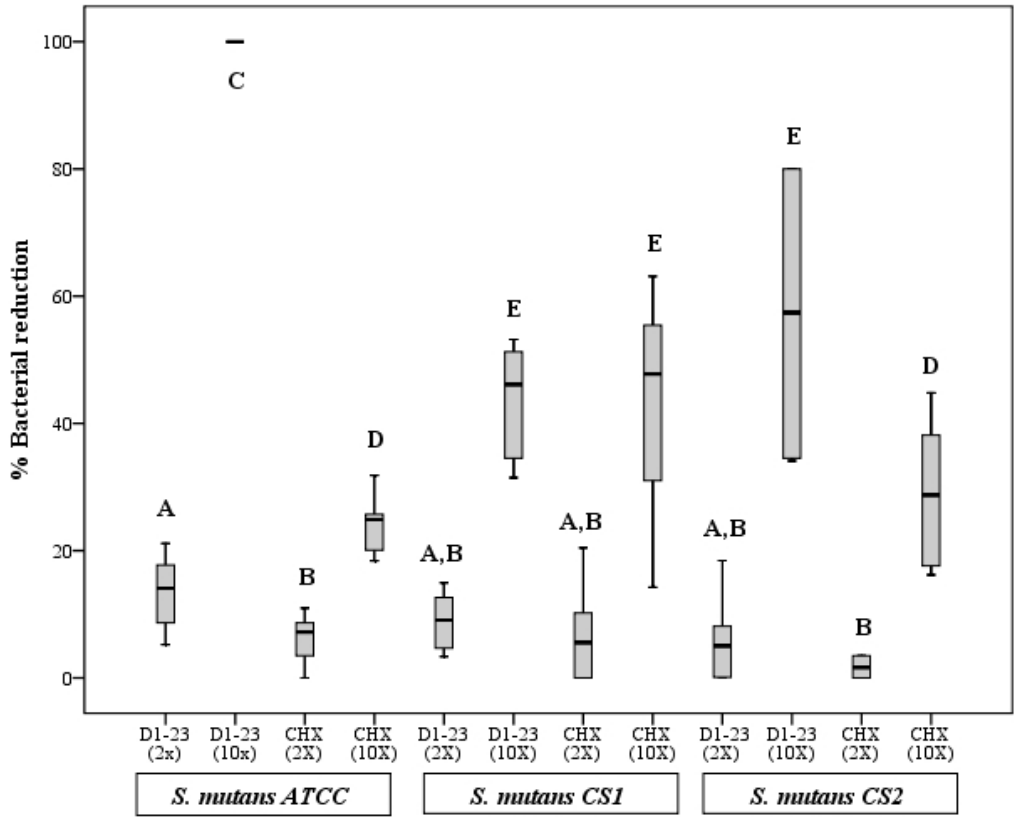

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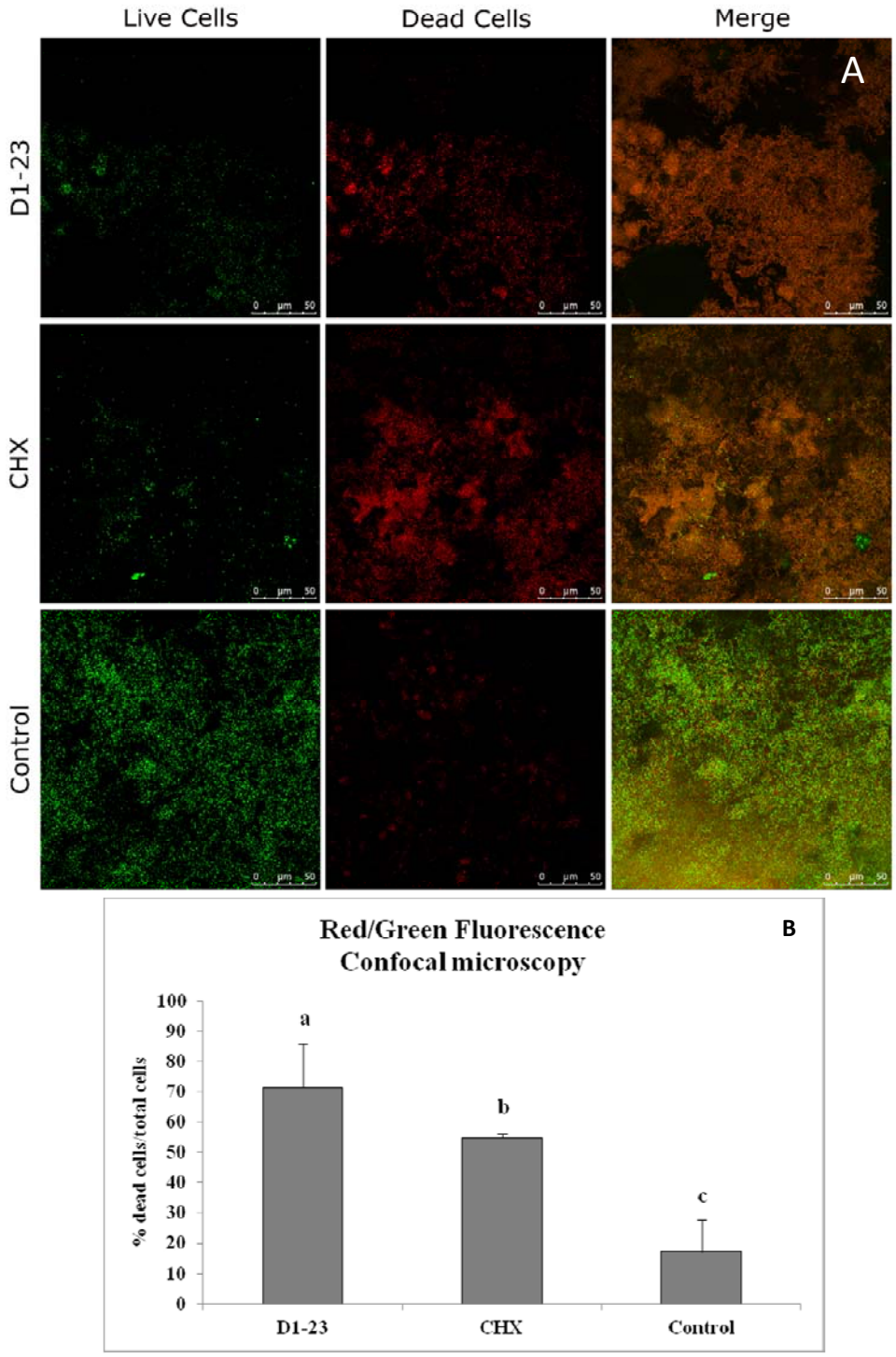

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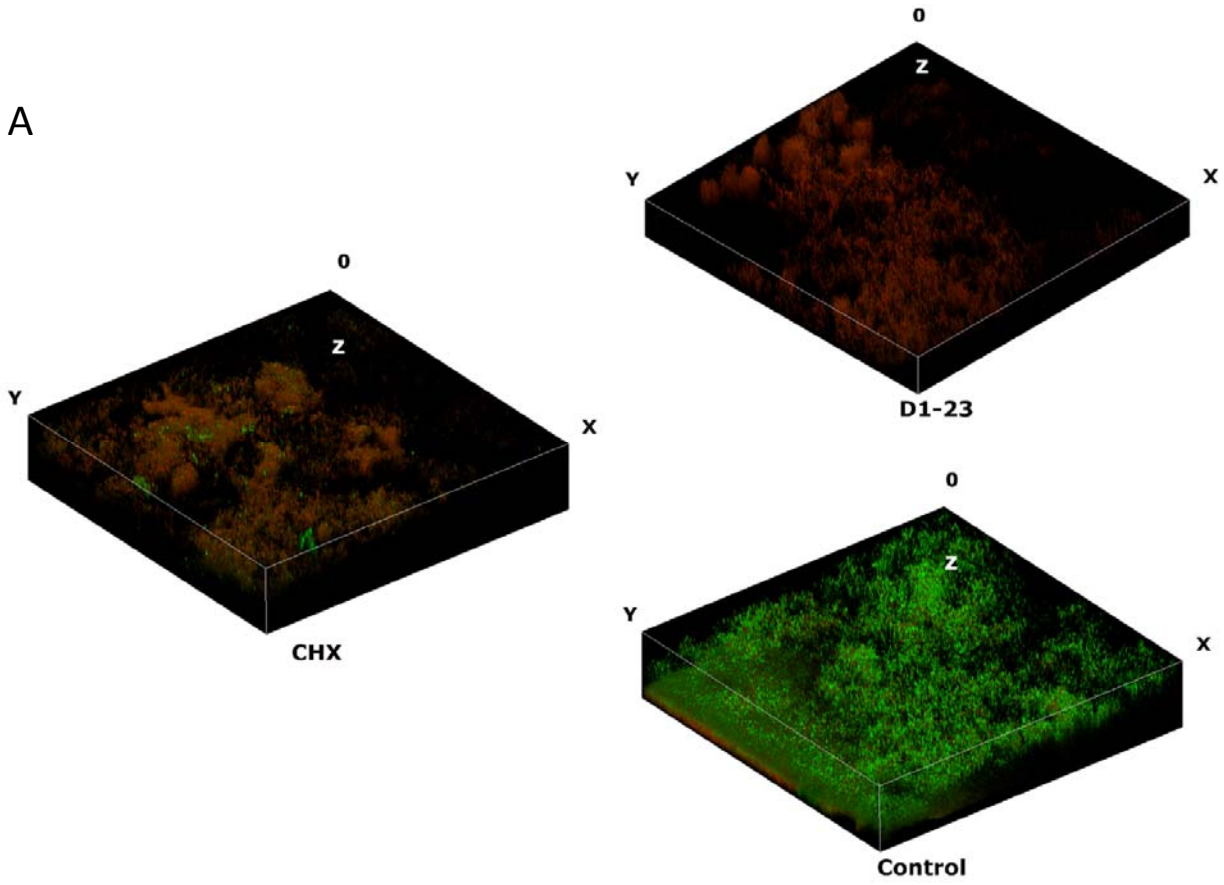

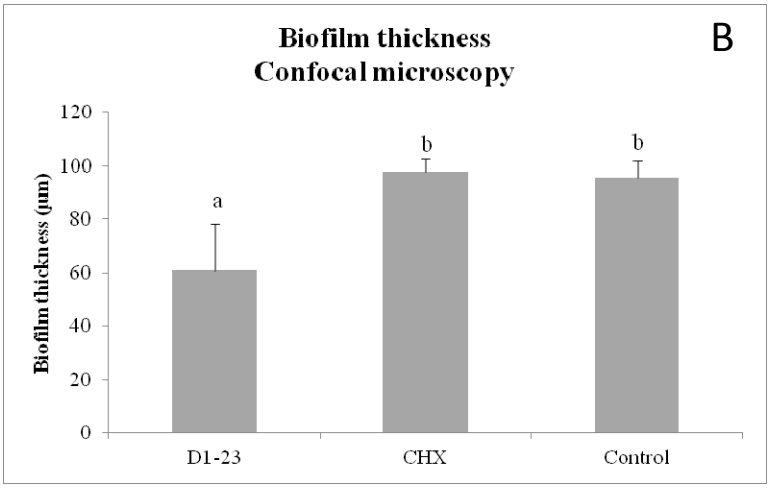

Figure 6. Biofilm thickness. **A.** Representative 3D images obtained by CLSM of *S. mutans* biofilms after D1-23 and CHX exposure for 24h. **B.** Means (bars-standard deviations) of biofilm thickness obtained after CLSM analysis of S. mutans biofilm.

[a] Different lower case letter show statistical differences among the groups, according to ANOVA and Tukey tests ($p<0.05$).

Referências Bibliográficas Gerais

1. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Microsc. 2003 Dec;36(4):247-52.

2. American Academy of Pediatric Dentistry. Reference manual: guidelines for pulp therapy for primary and young permanent teeth. 2009. http://www.aapd.org/media/Policies_Guidelines/G_Pulp.pdf.

3. Arai T, Ochiai K, Senpuku H. *Actinomyces naeslundii* GroEL-dependent initial attachment and biofilm formation in a flow cell system. J Microbiol Methods. 2015 Feb;109:160-6.

4. Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol. 2002 Mar;40(3):1001-9.

5. Bals R, Wilson JM. Cathelicidins – a family of multifunctional antimicrobial peptides. Cell Mol Life Sci. 2003 Apr;60(4):711-20.

6. Chairatana P, Chu H, Castillo PA, Shen B, Bevins CL, Nolan EM. Proteolysis Triggers Self-Assembly and Unmasks Innate Immune Function of a Human α-Defensin Peptide. Chem Sci. 2016 Mar 1;7(3):1738-1752. Epub 2015 Dec 10.

7. Colak H, Dulgergil CT, Dalli M, Hamidi MM. Early childhood caries update: A review of causes, diagnoses, and treatments. J Nat Sci Biol Med. 2013 Jan;4(1):29-38.

8. Colombo NH, Ribas, LF, Pereira JA, Kreling PF, Kressier CA, Tanner AC, Duque C. Antimicrobial peptides in saliva of children with severe early childhood caries. Arch Oral Biol. 2016 Sep;69:40-6.

9. Dale BA, Fredericks LP. Antimicrobial peptides in the oral environment: expression and function in health and disease. Curr Issues Mol Biol. 2005 Jul;7(2):119-33.

10. Dale BA, Tao R, Kimball JR, Jurevic RJ. Oral antimicrobial peptides and biological control of caries. BMC Oral Health. 2006 Jun 15;6 Suppl 1:S13.

11. Davidopoulou S, Diza E, Menexes G, Kalfas S. Salivary concentration of the antimicrobial peptide LL-37 in children. Arch Oral Biol. 2012 Jul;57(7):865-9.

12. De Yang, Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., Oppenheim, J. J., Chertov, O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med. 2000 Oct 2;192(7):1069-74.

13. Dietrich DE, Martin AD, Brogden KA. Human beta-defensin HBD3 binds to immobilized Bla g2 from the German cockroach (Blattella germanica). Peptides. 2014 Mar;53:265-9.

14. Drury TF, Horowitz AM, Ismail AI, Maertens MP, Rozier RG, Selwitz RH. Diagnosing and reporting early childhood caries for research purposes. A report of a workshop sponsored by the National Institute of Dental and Craniofacial Research, the Health Resources and Services Administration, and the Health Care Financing Administration. J Public Health Dent. 1999 Summer;59(3):192-7.

15. Duque C, Negrini TC, Sacono NT, Boriollo MFG, Hofling JF, Hebling J, Spolidorio DMP. Genetic polymorphism of Streotococcus mutans strains associated with incomplete caries removal. Braz J Oral Sci. 2009;8(1): 2-8.

16. Fejerskov O, Kidd E. Cárie Dentária. A doença e seu tratamento clínico. Editora Santos, 2005.

17. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. J Biol Chem. 1992 Nov 15;267(32):23216-25.

18. Isong I, Dantas L, Gerard M, Kuhlthau K. Oral Health Disparities and Unmet Dental Needs among Preschool Children in Chelsea, MA: Exploring Mechanisms, Defining Solutions. J Oral Hyg Health. 2014;2.

19. Lemos JA, Abranches J, Burne RA. Responses of cariogenic streptococci to environmental stresses. Curr Issues Mol Biol. 2005;7: 95-107.

20. Low W, Tan S, Schwartz S. The effect of severe caries on the quality of life in young children. Pediatr Dent. 1999 Sep-Oct;21(6):325-6.

21. Lygidakis NA, Marinou D, Katsaris N. Analysis of dental emergencies presenting to a community paediatric dentistry centre. Int J Paediatr Dent. 1998 Sep;8(3):181-90.

22. Mattos-Graner RO, Correa MSNP, Latorre MRO, Peres RCR, Mayer MPA. Mutans streptococci oral colonization in 12-30-month-old Brazilian children over a one year follow-up period. J Public Health Dent. 2001 Summer;61(3):161-7.

23. Mccormick TS, Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. Periodontol 2000. 2010 Oct;54(1):195-206.

24. Mohebbi SZ, Virtanen JI, Vahid-Golpayegani M, Vehkalahti MM. Feeding habits as determinants of early childhood caries in a population where prolonged

breastfeeding is the norm. Community Dent Oral Epidemiol. 2008 Aug;36(4):363-9.

25. Nakamura K, Sakuragi N, Takakuwa A, Ayabe T. Paneth cell α-defensins and enteric microbiota in health and disease. Biosci Microbiota Food Health. 2016;35(2):57-67.

26. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M.Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, {beta}-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother. 2005 Jun;55(6):888-96.

27. Palmer RJ, Jr., Gordon SM, Cisar JO, Kolenbrander PE. Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. J Bacteriol. 2003 Jun;185(11):3400-9.

28. Phattarataratip E, Olson B, Broffitt B, Qian F, Brogden KA, Drake DR, Levy SM, Banas JA. *Streptococcus mutans* strains recovered from caries-active or caries-free individuals differ in sensitivity to host antimicrobial peptides. Mol Oral Microbiol. 2011 Jun;26(3):187-99.

29. Ramos-Gomez FJ, Weintraub JA, Gansky SA, Hoover CI, Featherstone JD. Bacterial, behavioral and environmental factors associated with early childhood caries. J Clin Pediatr Dent. 2002 Winter;26(2):165-73.

30. Reynolds NL, De Cecco M, Taylor K, Stanton C, Kilanowski F, Kalapothakis J, Seo E, Uhrin D, Campopiano D, Govan J, Macmillan D, Barran P, Dorin JR. Peptide fragments of a beta-defensin derivative with potent bactericidal activity. Antimicrob Agents Chemother. 2010 May;54(5):1922-9.

31. Sansone C, van Houte J, Joshipura K, Kent R, Margolis HC. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res. 1993 Feb;72(2):508-16.

32. Saxena D, Li Y, Caufield PW. Identification of unique bacterial gene segments from Streptococcus mutans with potential relevance to dental caries by subtraction DNA hybridization. J Clin Microbiol. 2205;16:204-211.

33. SBBrasil. Brazilian Oral Health Report 2010. [May 30th, 2016]; Available from: http://dab.saude.gov.br/CNSB/sbbrasil/arquivos/projeto_sb2010_relatorio_final.pdf

34. Scott, M. G., Davidson, D. J., Gold, M. R., Bowdish, D., Hancock, R. E. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. J Immunol. 2002 Oct 1;169(7):3883-91.

35. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011 Mar;90(3):294-303.

36. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, Wells N, Berndt J, Dale BA. Salivary antimicrobial peptide expression and dental caries experience in children. Antimicrob Agents Chemother. 2005 Sep;49(9):3883-8.

37. Vadiakas G. Case definition, aetiology and risk assessment of early childhood caries (ECC): a revisited review. Eur Arch Paediatr Dent. 2008 Sep;9(3):114-25.

38. van Houte J, Lopman J, Kent R. The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J Dent Res. 1996 Apr;75(4):1008-14.

39. van Houte J, Lopman J, Kent R. The predominant cultivable flora of sound and carious human root surfaces. J Dent Res. 1994 Nov;73(11):1727-34.

40. van Houte J, Sansone C, Joshipura K, Kent R. *In vitro* acidogenic potential and mutans streptococci of human smooth-surface plaque associated with initial caries lesions and sound enamel. J Dent Res. 1991 Dec;70(12):1497-502.

41. Wiesner J, Vilcinskas A. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 2010 Sep-Oct;1(5):440-64.

42. Wu Z, Hoover DM, Yang D, Boulègue C, Santamaria F, Oppenheim JJ, Lubkowski J, Lu W. Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human betadefensin 3. Proc Natl Acad Sci U S A. 2003 Jul 22;100(15):8880-5.

43. Yang D, Biragyn A, Hoover D M, Lubkowski J, Oppenheim J J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu Rev Immunol. 2004;22:181-215.

44. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schröder JM, Wang JM, Howard OM, Oppenheim JJ. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science. 1999 Oct 15;286(5439):525-8.

45. Zasloff M. Antimicrobial peptides and suppression of apoptosis in human skin. J Invest Dermatol. 2009 Apr;129(4):824-6.

46. Zhang Z, Cherryholmes G, Shively JE. Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin. J Leukoc Biol. 2008 Sep;84(3):780-8.

Anexos

Anexo A

Aprovação do Comitê de Ética e Pesquisa em Animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Relatório Final do trabalho intitulado **"Avaliação do potencial antimicrobiano e citotoxicidade de fragmentos peptídicos catiônicos isolados ou combinados na prevenção da cárie dentária"**, Processo FOA nº 2013-00198, sob responsabilidade de Cristiane Duque e colaboração de Natália Helena Colombo, Lais Fernanda Fonseca Ribas, Paula Fernanda Kreling, Kelly Limi Aida, Luis Mateus de Almeida Matias, Loiane Massunari, Célio Percinoto, Eduardo Maffud Cilli e Norival Alves Santos Filho foi aprovado pela CEUA em 09 de Dezembro de 2015.

CERTIFICATE

We certify that the study entitled **"Evaluation of antimicrobial potential and cytotoxicity of cationic peptide fragments alone or in combination in the prevention of dental caries"**, Protocol FOA nº 2013-00198, under the supervision of Cristiane Duque and collaboration of Natália Helena Colombo, Lais Fernanda Fonseca Ribas, Paula Fernanda Kreling, Kelly Limi Aida, Luis Mateus de Almeida Matias, Loiane Massunari, Célio Percinoto, Eduardo Maffud Cilli and Norival Alves Santos Filho had its the Final Report approved by the CEUA on December 09, 2015.

Profa. Adj. Maria Cristina Rosifini Alves Rezende
Vice-Coordenadora da CEUA
CEUA Vice-Coordinator

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Apêndices

Apêndice A

Protocolo de Citotoxicidade Celular

1) Realizar o crescimento celular com escolha da linhagem de células pertinentes ao experimento

2) Após conferência do crescimento celular realizar estimulação celular com tripsina e manter à 37°C em estufa de CO_2 à 5% por 5 minutos;

- Adicionar 0,3 mg/ml de tripsina inibitória para inativar a protease e centrifugar por 5 minutos a 500xg;

- Suspender em meio fresco e distribuir 200µl por poço em uma placa de 96 poços;

- Incubar overnight à 37°C em estufa de CO_2 à 5% para adesão celular antes da estimulação;

- Estimular as células com os agentes antimicrobianos teste e controles negativo e positivo;

- Incubar a placa por 24h à 37°C em estufa de CO_2 à 5%.

3) Após 24h avaliar a viabilidade celular com ensaio de colorimetria MTT;

- Aspirar o meio cuidadosamente e acrescentar 90µl de DMEM e 10µl de MTT;

- Aspirar novamente e acrescentar 100µl de solução de ácido isopropanol;

- Transferir para dois novos poços alíquotas de 50µl;

- Avaliar a viabilidade celular usando espectofotometro com absorvencia de 570nm.

3.1. Coloração DAPI (4,6-Diamidino-2-phenylindole dihydrochoride 4,6-Diamidino-2-phenylindole dihydrochoride)

- Aspirar o meio cuidadosamente e acrescentar 100µl de DAPI por 5 minutos;

- Aspirar novamente e lavar com soro fisiológico;

- Avaliar a fragmentação nucleica usando microscopia de fuorescencia.

Apêndice B

Protocolo de Determinação da CIM e CBM

Dia 1: Reativar o microrganismo em BHI agar para as bactérias (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

Dia 2: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 UFC em 5ml) e incubar.

Dia 3: Diluição dos antimicrobianos teste:

*Pipetar 50µl do peptídeo na concentração de 2mg/ml no primeiro e segundo poço de uma placa de 96 poços;

*Pipetar 50µl de água em cada poço, exceto o primeiro;

*Ressuspender o segundo poço e transferir 50µl para o terceiro poço, e assim sucessivamente (diluição seriada);

*Tampar a placa, cobrir com papel alumínio e manter na geladeira.

- Preparar o inoculo de acordo com a curva de crescimento: D.O. 0,5 para as bactérias.

*Mueller-Hinton caldo para as bactérias;

*Diluir 1000x no meio de cultura 2x concentrado (500µl do inoculo em 4,5ml de meio – 3 tubos).

- Ressuspender 50µl do inoculo em cada poço; tampar a placa e incubar por 24h.

Dia 4: Pipetar 15µl de solução de Resazurina 0,01% em cada poço e incubar por 4h.

- Plaquear o último poço com coloração azulada (CIM) e, no mínimo, 2 poços anteriores para se determinar a CBM.

Dia 5: Leitura das placas.

Apêndice C

Preparo dos corpos de prova

Obtenção dos corpos de prova: dentes incisivos centrais inferiores permanentes de bovinos com idade entre 2 e 3 anos, mantidos em recipientes plásticos com solução de formol a 2% pH 7,0 durante 1 mês.

Confecção dos corpos de prova: Blocos de esmalte bovino (2mm x 2mm x 2mm) da porção mais plana da vestibular das coroas;

- Após a obtenção dos blocos de esmalte, ajustar a dentina para obtenção de superfícies paralelas entre esmalte e dentina (espessura ± 2 mm);

- Fixar os blocos em discos de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 8 mm de espessura), com auxílio de cera pegajosa, com a superfície dentinária voltada para cima, levar este conjunto à politriz BETA – grinder polisher;

- Desgastá-los com lixas de granulação 320, peso de 2 lbs, durante 30 segundos sob baixa rotação e refrigeração;

- Remover os blocos e fixá-los novamente com a superfície do esmalte voltada para cima e polir com a sequência: lixa de granulação 600, 4 lbs, tempo de 30 segundos, refrigeração a água; lixa de granulação 1200, 4 lbs, 30 segundos, refrigeração a água. Entre cada polimento, submeter os corpos de provas ao ultra-som, em água deionizada durante 2 minutos;

- Polir o esmalte com papel feltro para polimento e suspensão de diamante, 4 lbs, por 1 minuto. Lavar os corpos de provas com jato de água deionizada durante 30 segundos e submetê-los ao ultra-som, durante 2 minutos, imersos em solução de limpeza diluída na proporção 20:1 em água destilada. (Manter os blocos em ambiente umedecido com formol 2% pH 7,0).

Leitura em goniômetro: ler os corpos de prova em aparelho goniômetro, com energia livre variando entre média e desvio padrão de 114±15 e dividí-los randomicamente em três grupos (n=6).

Apêndice D

Protocolo do Ensaio de Biofilme

Dia 1: Reativar o microrganismo em BHI agar para as bactérias (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

Dia 2: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 UFC em 5ml) e incubar.

Dia 3: Preparar a placa de 96 poços com fundo em U (não tratada): pipetar 200µl de saliva estimulada (esterilizada por uma membrana de 0,22µm) em cada poço e incubar por um período de 4h;

- Preparar o inoculo de acordo com a curva de crescimento: D.O. 0,5 para todos os microrganismos teste;

- Remover a saliva com ponteira estéril e acrescentar 10µl do inoculo e 90µl de BHI suplementados com 0,5% de glicose; incubar por 48h.

Dia 5: Remover o sobrenadante com ponteira estéril, e lavar os poços com 200µl de solução salina 0,9% durante 3 minutos, remover toda a solução com ponteira estéril;

- Acrescentar 100µl do antimicrobiano na concentração 2 e 10 vezes maiores que a CBM pré-determinada;

    *Incluir as mesmas concentrações para os controles positivos (Clorexidina);

    *Cada concentração deverá ser testada em 3 poços por experimento (triplicata);

    *Deixar três poços com 200µl de água destilada estéril (controle negativo);

- Incubar por 24h.

Dia 6: Com o auxílio de pinças estéreis remover o corpo de prova, mergulha-lo em eppendorfe com solução salina estéril, passar para um novo eppendorfe com solução salina estéril e finalmente para um novo eppendorfe também com solução salina estéril;

- Levar ao sonicador com 30 de frequência por 30s;

-Diluição e plaqueamento de todos os eppendorfes.

Dia 7: Leitura das placas.

Apêndice E

Microscopia Confocal

Dia 1: Reativar o microrganismo em BHI agar para as bactérias (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

Dia 2: Preparar a placa de vidro de 96 poços: acrescentar 100µl BHI estéril em cada poço;

- Repicar o microrganismo da placa para solução salina até atingir a DO estabelecida para cada microorganismo;

- Acrescentar o microorganismo aos poços contendo BHI. (quantidade varia conforme cepa bateriana);

- Incubar a placa em anaerobiose a 37°C por 24h para formação de biofilme.

Dia 3: Remover o sobrenadante com ponteira estéril, e lavar os poços cuidadosamente com 200µl de solução salina 0,9% por duas vezes, remover toda a solução com ponteira estéril;

- Acrescentar 30µl do antimicrobiano na concentração 10 vezes maior que a CBM pré-determinada;

- Deixar o antimicrobiano agir por um periodo de 2 horas e manter a placa em condição de anaerobiose;

*Incluir as mesmas concentrações para os controles positivos (Clorexidina);

*Cada concentração deverá ser testada em 3 poços por experimento (triplicata);

*Deixar três poços com 200µl de água destilada estéril (controle negativo);

- Lavar os poços cuidadosamente com 200µl de solução salina 0,9% por duas vezes, remover toda a solução com ponteira estéril;

- Adicionar a solução reativa para viabilidade celular e deixar agir por 15 minutos;

- Lavar os poços cuidadosamente com 200µl de solução salina 0,9% por duas vezes, remover toda a solução com ponteira estéril;

- Levar a placa para leitura em microsópio eletrônico;

- Análise das imagens e contabilização de bactérias vivas e mortas.

Apêndice F

Guide for Authors (Biofouling - The Journal of Bioadhesion and Biofilm Research)

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