UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Midian Clara Castillo Pedraza

Modulação de genes e de seus produtos afeta a virulência de *Streptococcus mutans*

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Orientador: Dra. Marlise Inêz Klein Furlan

Coorientador: Prof. Dr. Pedro Luiz Rosalen

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Comissão julgadora

Tese para obtenção do grau de Doutor em Reabilitação Oral

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RESUMO

Streptococcus mutans orquestra a formação de biofilmes cariogênicos através da produção da matriz extracelular que contém exopolissacarídeos (EPS), DNA extracelular (eDNA) e ácidos lipoteicóicos (LTA). EPS são um marcador de virulência para cárie, mas não está claro como os genes associados com eDNA (*lytS* e *lytT*) e LTA (*dltA* e *dltD*) afetam a virulência de *S. mutans.* Portanto, avaliou-se como os genes *lytST*, *dltAD* e *gtfB* (EPS-insolúveis) afetariam o desenvolvimento de lesões cariosas em ratos e a sobrevivência de larvas (*Galleria mellonella*) após infecção sistêmica e para esclarecer sua contribuição na patogenicidade dessa bactéria. Ainda, avaliou-se o efeito dos tratamentos tópicos com miricetina (afeta a síntese de EPS), composto 1771 (modula o metabolismo do LTA) e flúor (prevenção da cárie) sobre biofilmes in vitro de *S. mutans.* Alimentou-se os ratos com dieta cariogênica e inoculou-se com cepa parental UA159 e cepas com deleção de genes Δ*lytS*, Δ*dltD* e Δ*gtfB* (n=14). Após 5 semanas, avaliou-se as populações microbianas (cultivável total e *S. mutans*) e as lesões de cárie. Injetou-se a cepa parental e as cepas com deleção de genes Δ*gtfB*, Δ*lytS*, Δ*lytT*, Δ*dltA* e Δ*dltD* na hemocele de larvas e registrou-se a sobrevivência das larvas longitudinalmente (n=10). Formou-se biofilmes de *S. mutans* sobre discos de hidroxiapatita revestidos com película salivar e realizou-se tratamentos tópicos duas vezes ao dia: miricetina (Mir), composto 1771, flúor (F) Mir+1771, Mir+F, 1771+F, Mir+1771+F e veículo (controle) (n=6). Determinou-se nesses biofilmes diversos parâmetros: biomassa, população, componentes da matriz (EPS solúveis e insolúveis em água, eDNA e LTA), arquitetura tridimensional e expressão gênica. Ainda, avaliou-se a citotoxicidade dos tratamentos (n=8). Na análise da população microbiana em ratos, a proporção de *S. mutans* na microbiota total recuperada foi maior para o grupo UA159 *versus* os grupos com deleção (15, 3 e 6 vezes para Δ*gtfB*, Δ*lytS* e Δ*dltD,* respectivamente). UA159 produziu maior número de lesões de cárie, com maior severidade (lesões em dentina): no esmalte (50% mais lesões) e na dentina (≥80% mais cavidades) de superfícies lisas e superfície sulcal (≅30% mais lesões em esmalte e ≥60% mais lesões em dentina) ao ser comparada com as cepas com deleção. A sobrevivência de *G. mellonella* foi menor em larvas infectadas com UA159, seguido de Δ*gtfB* quando comparado com as outras cepas com deleção gênica. Tratamentos com miricetina e composto 1771 isolados resultaram em biofilmes com menores contagens de *S. mutans*, biomassa, EPS solúveis e insolúveis (*vs*. veículo). Entretanto, a combinação Mir+1771+F foi mais eficaz, pois diminuiu a contagem de *S. mutans* (3 logs), biomassa (60%), EPS solúveis (74%) e insolúveis (87,5%) (*vs*. veículo). Ainda, essa estratégia afetou negativamente eDNA, LTA, alterou o tamanho das microcolônias e inibiu a expressão dos genes *gtfBC*. Adicionalmente, os tratamentos Mir+1771 e Mir+F (*vs*. controle de viabilidade) foram levemente

citotóxicos (redução de viabilidade celular em 13% e 25%, respectivamente). Portanto, os genes *lytST*, *dltAD* e *gtfB* contribuem para a cariogenicidade e a virulência de *S. mutans*; a estratégia com miricetina e composto 1771 modula os produtos destes genes, tornando-a promissora para o controle desses biofilmes.

Palavras chave: Cárie dentária. *Streptococcus mutans.* Placa dentária.

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ABSTRACT

Streptococcus mutans orchestrates the cariogenic biofilms formation through the production of an extracellular matrix that contains exopolysaccharides (EPS), extracellular DNA (eDNA), and lipoteichoic acids (LTA). EPS is a virulence marker of dental caries, but it is unclear how genes associated with eDNA (*lytS* and *lytT*) and LTA (*dltA* and *dltD*) affect *S. mutans* virulence. Therefore, this study evaluated how the genes *lytST*, *dltAD* and *gtfB* (insoluble EPS) affected the development of carious lesions in rats and the survival of larvae (*Galleria mellonella*) after systemic infection to clarify its contribution to the pathogenicity of *S. mutans*. Also, it assessed the effect of topical treatments with myricetin (affects EPS synthesis), compound 1771 (modulates LTA metabolism) and fluoride (caries prevention) on *in vitro S. mutans* biofilms. The rats received a cariogenic diet and were inoculated with the parental strain UA159 and its strains with deletion of genes Δ*lytS*, Δ*dltD,* and *ΔgtfB* (n=14). After five weeks, viable microbial populations (total cultivable and *S. mutans*) and caries lesions were evaluated. Larvae were inoculated by intra-hemocoel injection with the parental strain and deletion strains *ΔgtfB*, *ΔlytS*, *ΔlytT*, *ΔdltA*, and *ΔdltD*, and larval survival was recorded longitudinally (n=10). *S. mutans in vitro* biofilms were formed on saliva-coated hydroxyapatite discs and topically treated twice-daily: Myricetin (Myr), Compound 1771, Fluoride (F), Myr+1771, Myr+F, 1771+F, Myr+1771+F, and vehicle-control (n=6). The biofilms were evaluated to determine biomass, viable *S. mutans* counts, matrix components (water-soluble and -insoluble EPS, eDNA, and LTA), tridimensional architecture, and gene expression. Also, the cytotoxicity of the treatments was evaluated. In the rodent caries model, the proportion of *S. mutans* in total microbiota recovered was higher for the UA159 group *versus* the deletion groups (15, 3 and 6-fold for *ΔgtfB*, Δ*lytS,* and *ΔdltD*, respectively). The parental strain UA159 yielded higher number of caries lesions that were also more severe (lesions in dentin): on enamel (50% more lesions) and dentin (≥80% more cavities) of smooth surfaces, and on enamel (≅30% more lesions) and dentin (≥60% more lesions) sulcal surfaces when compared to the deletion strains. The *G. mellonella* survival was lower in UA159-infected larvae, followed by *ΔgtfB*-infected when compared to other gene deletion strains. Topical treatments with either myricetin or compound 1771 alone led the biofilms with lower amounts of *S. mutans* viable counts, biomass, water-soluble, and water-insoluble EPS (*vs.* vehicle). However, the combination Myr+1771+F was more efficient, as it decreased 3 logs of *S. mutans* counts, ≅60% of the biomass, 74% of % water-soluble EPS, and 87.5% of water-insoluble EPS counts (*vs.* vehicle). Also, this strategy negatively affected eDNA, LTA, altered the size of microcolonies, and inhibited the expression of *gtfBC* genes. Additionally, Mir+1771 and Mir+F (25%) treatments (*vs*. viability control) were mildly cytotoxic (reduction of viability by 13% and

25%, respectively). Therefore, the genes *lytST*, *dltAD,* and *gtfB* contribute to the cariogenicity and virulence of *S. mutans*; a strategy with myricetin and compound 1771 modulates the products of these genes, making it a promissory approach to control cariogenic biofilms.

Keywords: Dental caries. *Streptococcus mutans*. Dental biofilm.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária acontece pela constante produção de metabólitos microbianos (ácido orgânicos), que acidificam o biofilme e afetam os cristais de hidroxiapatita (desmineralização)[1], que é o principal componente no esmalte. A desmineralização é considerada "normal" quando é alternada com a remineralização (íons de cálcio e fosfato na saliva ajudam a evitar a perda de tecido mineralizado) com o objetivo de neutralizar os processos degenerativos da cárie[2,3,4]. Quando a desmineralização supera este processo dinâmico, aparecem lesões cariosas irreversíveis que, se não tratadas adequadamente, podem levar ao detrimento dentário.

Interações entre substrato (dente), dieta rica em carboidratos e biofilme microbiano[1,5,6,7] são necessárias para a ocorrência da cárie. Além disso, outros fatores podem interagir e contribuir com a doença, como os fatores ambientais dentro da cavidade bucal (higiene, fluxo salivar, pH, proteínas, $Ca^{2+,}$ PO_4^{3-}) e fatores pessoais do indivíduo (renda, perfil sócio - demográfico, acesso à saúde dental, atitudes)[1,8]. A cárie pode afetar crianças e adultos sem distinção de sexo ou cultura, tornando-se um problema de saúde pública mundial[9,10].

A cárie pode não apresentar sintomas, mas na maioria dos eventos avançados (com presença de cavidades), o indivíduo sente desconforto e dor intensa, acompanhado de inflamação na polpa e/ou abcesso dentário, tornando a pessoa incapaz para exercer atividades diárias[11]. Neste caso, o tratamento pode ser endodôntico ou exodontia. A perda dentária pode gerar baixa autoestima, problemas na fonética, dificuldade no processamento dos alimentos e na interação social.

Estratégias de prevenção da cárie incluem a escovação diária com dentifrício contendo flúor (mínimo três vez ao dia)[12], a utilização do fio dental (mínimo uma vez ao dia), restrição de açúcares na dieta e consultas com o cirurgião-dentista para profilaxia dentária (cada 6 meses), aplicação de flúor e selantes[11]. Entretanto, com todas essas estratégias, a cárie continua sendo considerada a doença mais prevalente no mundo. Segundo *Global Burden of Disease Study* (GBD) 1990 - 2016, as doenças na cavidade bucal afetam a metade da população mundial (3580 milhões), sendo a cárie a mais prevalente entre elas[13]. Estima-se que, no mundo, 2400 milhões de pessoas apresentam cárie em dentes permanentes e 489 milhões de crianças sofrem de cáries nos dentes decíduos[13].

Existem aproximadamente 700 espécies de microrganismos que habitam a cavidade bucal[14], onde *Streptococcus mutans* desempenha um papel importante na etapa de transição da forma não-patogênica para biofilmes altamente cariogênicos[15]. Embora existam outros microrganismos que apresentem a capacidade de produzir ácido e viver no ácido, *S. mutans* destaca-se pela sua capacidade de produzir uma matriz extracelular rica em exopolissacarídeos[16]. *S. mutans* são cocos Gram positivos, anaeróbios facultativos; e tem sido estudado por meio de diversas técnicas bioquímicas, sorológicas e genéticas.

Os carboidratos disponíveis na dieta diária favorecem a formação de um biofilme altamente coesivo. Os microrganismos utilizam a sacarose para modular a construção da matriz extracelular mediante as frutosiltransferases (Ftfs) e glicosiltransferases (Gtfs), que sintetizam exopolissacarídeos (EPS)[15,17-20] Ainda, a presença de amido aumenta o poder cariogênico da sacarose[22,23]. A ingestão frequente desses carboidratos intercalados aumenta a aderência de *S. mutans* e demais microrganismos sobre a superfície de hidroxiapatita[24], favorecem a produção ácida dentro do biofilme[20,25] e proporciona estabilidade e integridade à matriz extracelular[19,26-28].

A capacidade de *S. mutans* para formar biofilmes com uma matriz extracelular rica em EPS é significativa para a ocorrência da cárie[29], mas também existe relação com casos de bacteremia e de endocardite infecciosa (EI) com o desenvolvimento de biofilme formado por esse microrganismo[30,31]. EI é uma infecção no endocárdio normalmente acontece quando uma bactéria de outra parte do corpo, como as encontradas na cavidade bucal, se dissemina pela corrente sanguínea alojando-se nas áreas danificadas do revestimento interno do coração (endocárdio). As bactérias aproveitam as condições favoráveis de crescimento para deposição e formação de biofilme[32]. Independentemente do lugar da formação do biofilme, todo biofilme tem pelo menos uma propriedade em comum, a presença de uma matriz derivada de microrganismos[33].

Matriz extracelular é considerada como uma barreira de proteção contra substâncias nocivas que impedem o desenvolvimento do biofilme[33]. Matriz extracelular de biofilmes cariogênicos tem os EPS como componentes indispensáveis para a sua construção e estruturação tridimensional (3D)[19,33,34]. *S. mutans* é um grande produtor de substâncias poliméricas extracelulares incluindo EPS, DNA

extracelular (eDNA) e ácido lipoteicóicos (LTA)[16,24,33,34]. Esses componentes encontrados na matriz, são importantes para sua construção[24]. Ainda, são identificados dentro da matriz de biofilmes orais outros componentes: proteínas, lipídios e lipopolissacarídeos (derivados de espécies Gram negativas)[35-37].

O eDNA fornece integridade estrutural e estabilidade nos biofilmes de *S. mutans*[23]. Este componente da matriz pode ser liberado durante o processo de lise celular e morte celular ou em algum momento pode também ser secretado por microvesículas ligadas à membrana[38]. A expressão dos genes *lytST, lrgAB* e *cidAB* de *S. mutans* estão relacionados com a presença de eDNA na matriz, quando os carboidratos da dieta e as condições ambientais são favoráveis[23,39]. O sistema *lrgAB* / *cidAB* é importante na formação do biofilme, na tolerância ao estresse oxidativo e na regulação da autólise; estas funções são reguladas pelo sistema de dois componente *lytST* através da disponibilidade de carboidrato via a proteína *ccpA* (do inglês *catabolite control protein* A)[39,40].

O LTA está associado à aderência de estreptococos orais[41,42] e desempenha um papel significativo na colonização de bactérias Gram positivas, contribuindo com a formação e a patogenicidade do biofilme[41,42]. LTA encontra-se ligado na membrana celular por uma lipoproteína e podem ser liberados para o ambiente extracelular[43-45], especialmente durante a remodelagem da parede celular. O metabolismo deste componente encontra-se associado à expressão dos genes do operon *dltABCD* e *SMU.775* de *S. mutans*[24,45,46]. Os genes de operon *dltABCD* adicionam os resíduos de D-alanina que favorecem a aderência das bactérias e a construção do biofilme[45,47,48]. O gene *SMU.775* (proteína hipotética) pode estar realizando a função do gene *LtaS* de *Staphylococcus aureus* e sintetizar o ácido lipoteicóico poliglicerol-fosfato a partir de fosfatidilglicerol[35,49].

Assim, uma pesquisa in vitro com *S. mutans* cepa parental UA159 e cepas com deleções de genes específicos utilizados para modular eDNA (Δ*lytS* e Δ*lytT*), LTA (Δ*dltA* e Δ*dltD*) e EPS insolúvel (Δ*gtfB*), avaliou a composição bioquímica da matriz[24]. Esse estudo detectou a presença de eDNA, LTA, EPS solúveis e insolúveis em água e proteínas solúveis na matriz de biofilmes monoespécie de *S. mutans* (cepa parental UA159 e cepas com deleção dos genes *gtfB*, *lytS, lytT, dltA* e *dltD)* e mistos (*S. mutans* – cepa parental UA159 ou mutante*, Streptococcus gordonii* e *Actinomyces naeslundii)*, avaliados em dois momentos 67 e 115 horas. A deleção de genes específicos afetou

não só a composição da matriz, mas também a dinâmica de populações bacterianas e a arquitetura 3D de biofilmes. O desenho experimental de biofilme in vitro utilizado, também mostrou que a ausência dos genes *lytS* e *lytT* induziram maior liberação de eDNA; o mesmo ocorreu para os genes *dltA* e *dltD* quanto a presença de LTA. Ainda, esses biofilmes mostraram aumento de EPS solúveis e insolúveis, que se incrementam ainda mais durante a maturação. Portanto, alterações nos processos de liberação de eDNA, LTA e EPS podem modificar a composição da matriz e interferir na formação do biofilme[24].

S. mutans possivelmente apresenta outros sistemas que são ativados para compensar a ausência de atividades dos sistemas *lytST* e *dltABCD* que também mantém as propriedades estruturais e funcionais da matriz do biofilme, pois era esperado que a deleção de *lytS* e *lytT* diminuísse a quantidade de eDNA e que a deleção de *dltA* e *dltD* reduzisse a quantidade de LTA na matriz (*versus* a cepa parental). No entanto, os dados mostraram mais eDNA e LTA em biofilmes com as respectivas cepas de deleção e a estrutura 3D foi distinta quando comparada à cepa parental[23]. Essas características poderiam ser por causa de alterações na remodelação e composição da parede celular (e carga)[39,40,47,50,51]. Porém, é imprescindível esclarecer minuciosamente as funções desses componentes, para compreender a fisiologia do biofilme e assim direcionar terapias para prevenir e/ou remover biofilmes cariogênicos.

Assim, essa pesquisa forneceu subsídios para a proposição de estudos mais detalhados do potencial de eDNA e LTA em conjunto com EPS na virulência de *S. mutans*. Além disso, este estudo avaliou agentes (com ação antimicrobiana) para reduzir o desenvolvimento de biofilme de *S. mutans*, interferindo nas funções da matriz. Investigar como esses genes influenciam na ocorrência e severidade de cárie ajudarão a entender melhor a biologia de *S. mutans* e a inibição das funções e ou composição da matriz pela aplicação de agentes específicos poderiam interferir nos processos de estruturação de biofilmes cariogênicos.

2 PROPOSIÇÃO

O objetivo geral desse trabalho foi avaliar a virulência de cepas de *S. mutans* com deleção dos genes *lytST* e *dltAD* em modelos in vivo de cárie e de infecção sistêmica, e testar agentes específicos para interferir na construção da matriz extracelular.

Os objetivos específicos foram:

Objetivo 1. Análise dos genes *lytS* e *dltD* de *S. mutans* no desenvolvimento e severidade de lesões cariosas in vivo utilizando um modelo animal (Ratos-fêmeas).

Objetivo 2. Avaliação das funções dos genes *lytST* e *dltAD* de *S. mutans* na virulência em infecções sistêmicas in vivo usando um modelo invertebrado (*Galleria mellonella*) e na resposta ao estresse oxidativo.

Objetivo 3. Avaliação do efeito da combinação de miricetina (interfere na síntese de exopolissacarídeos), composto 1771 (inibe o metabolismo de ácidos lipoteicóicos) e flúor (promove remineralização e interfere na acidogenicidade e aciduricidade) no desenvolvimento do biofilme de *S. mutans* in vitro.

3 PUBLICAÇÕES

Destaca-se que os objetivos 1 e 2 foram realizados e constam na Publicação 1 e que o objetivo 3 foi realizado e enviado para periódico científico como a Publicação 2. Essas duas publicações estão apresentadas a seguir.

3.1 Publicação 1*

Castillo Pedraza MC, Rosalen PL, de Castilho ARF, Freires IA, de Sales Leite L, Faustoferri RC, Quivey RG Jr, Klein MI. **Inactivation of *Streptococcus mutans* genes *lytST* and *dltAD* impairs its pathogenicity *in vivo*.** J Oral Microbiol. 2019; 11(1): 1607505. doi: 10.1080/20002297.2019.1607505.

Inactivation of *Streptococcus mutans* genes *lytST* and *dltAD* impairs its pathogenicity *in vivo*

ABSTRACT

Background: *Streptococcus mutans* orchestrates the development of a biofilm that causes dental caries in the presence of dietary sucrose, and, in the bloodstream, *S. mutans* can cause systemic infections. The development of a cariogenic biofilm is dependent on the formation of an extracellular matrix rich in exopolysaccharides, which contains extracellular DNA (eDNA) and lipoteichoic acids (LTAs). While the exopolysaccharides are virulence markers, the involvement of genes linked to eDNA and LTAs metabolism in the pathogenicity of *S. mutans* remains unclear. **Objective and Design:** In this study, a parental strain *S. mutans* UA159 and derivative strains carrying single gene deletions were used to investigate the role of eDNA (Δ*lytS* and Δ*lytT*), LTA (Δ*dltA* and Δ*dltD*), and insoluble exopolysaccharides (Δ*gtfB*) in virulence in a rodent model of dental caries (rats) and a systemic infection model (*Galleria mellonella* larvae). **Results:** Fewer carious lesions were observed on smooth and sulcal surfaces of enamel and dentin of the rats infected with Δ*lytS*, Δ*dltD*, and Δ*gtfB* (vs. the parental strain). Moreover, strains carrying gene deletions prevented the killing of larvae (vs. the parental strain). **Conclusions:** Altogether, these findings indicate that inactivation of *lytST* and *dltAD* impaired *S. mutans* cariogenicity and virulence *in vivo*.

* Artigo escrito seguindo as normas bibliográficas da revista J Oral Microbiology (ANEXO A). Disponível em: https://www.tandfonline.com/doi/full/10.1080/20002297.2019.1607505

Keywords: exopolysaccharides, eDNA, lipoteichoic acids, dental caries, systemic infection, oxidative stress

INTRODUCTION

Streptococcus mutans is considered a primary agent for dental caries development since it metabolizes dietary sugars into organic acids and produces exopolysaccharides, which trap acids in microniches within biofilms and at the interface between biofilms and tooth surfaces, causing tooth demineralization [1,2]. In addition, *S. mutans* can also cause systemic infections when it reaches the bloodstream (bacteremia) and persist in tissues such as the heart (endocarditis) [3-5]. In both cases, *S. mutans* uses its ability to form biofilms and to withstand environmental stresses (oxidative, nutritional, among others). Biofilms are communities of microbial cells structured and organized in a dynamic environment, covered and embedded within a three-dimensional (3D) extracellular matrix [6,7]. The matrix of cariogenic biofilms formed by *S. mutans* are rich in exopolysaccharides and contain extracellular DNA (eDNA) and lipoteichoic acids (LTA) that interact with exopolysaccharides [8]. While exopolysaccharides are proven cariogenic factors [2], it remains to be determined how genes associated with eDNA and LTA metabolism affect the occurrence of dental caries and systemic infection.

The presence of carbohydrates (sucrose and starch) contributes to the formation of the matrix in cariogenic biofilms. The matrix provides stability and structural integrity by allowing bacteria to adhere to tooth surfaces and provide protection against harmful environmental stimuli or other attacks [9]. The adhesion mechanism is guided by the glucosyltransferase (Gtfs) exoenzymes, which synthesize exopolysaccharides [9]. Furthermore, when carbohydrates are present in the oral cavity, acid production in the biofilm increases [10]. Organic acids can be retained in low pH niches within the biofilm because the exopolysaccharide-rich matrix prevents neutralization of these acids by saliva [11]. *S. mutans* encodes three different Gtfs, namely: GtfB, which produces water-insoluble exopolysaccharides (glucans); GtfC, which produces both water-soluble and -insoluble exopolysaccharides (glucans); and GtfD, which produces soluble exopolysaccharides (glucans); these enzymes are also known as Gtf-I, Gtf-SI, and Gtf-S, respectively [9].

In addition to exopolysaccharide production, *S. mutans* can release eDNA and LTA that will interact with exopolysaccharides [8,12]. Sucrose and starch induce the expression of *gtfB* [13,14], the two-component system *lytST* (associated with cell lysis and cell-wall remodeling) [13,14], and the operon *dltABCD* (related to LTA metabolism) [13,15]. This augmented expression increases the abundance of exopolysaccharides, eDNA and LTA that enable the construction of a bulky and sturdy extracellular matrix in *S. mutans* biofilms [8,14,16].

eDNA can be released during cell lysis and via microvesicles [17]. Reduction in *lytST* gene expression results in a decrease in eDNA presence in the matrix during the biofilm development process [14]. LTA is a cell wall polymer of Gram-positive bacteria, consisting of 1,3-polyglycerol-phosphate bound to the cell membrane by a lipoprotein, which during the remodeling of the cell wall or cell division, can be released into the extracellular environment [18,19]. LTA plays a significant role in the colonization of Gram-positive bacteria, contributing to the formation and pathogenicity of biofilms, and is associated with adherence and colonization of oral streptococci [20,21]. D-alanine promotes adhesion and biofilm development [22,23] and the *dltABCD* (D-alanyl-LTA) genes metabolize these residues during LTA synthesis [24]. Of note, activation of the *dltA* and *dltD* genes occurs during the early stages of matrix formation in mixed-species cariogenic biofilms [15].

In an *in vitro* study the *S. mutans* deletion strains Δ*lytS*, Δ*lytT*, Δ*dltA*, and Δ*dltD* contributed to an increased amount of extracellular eDNA and LTA in the matrix of biofilms, compared to either the parental *S. mutans* strain, UA159, or the Δ*gtfB* strain [8]. The presence of eDNA and LTA in the matrix resulted in an increased amount of soluble and insoluble exopolysaccharides, indicating that these biofilms could be more cariogenic (with a potential to cause increased caries) [8]. Thus, the evaluation of these genes associated with the essential components of the matrix (exopolysaccharides, eDNA and LTA) may help to clarify their contribution to the pathogenicity of *S. mutans*. Moreover, it is still unknown how these deletion strains behave when subjected to a systemic infection model. Thus, it is essential to clarify the functions of *lytST* and *dlt* genes to better understand the biology of *S. mutans* and to direct therapies to control biofilm formation by this species. The purpose of this research was to analyze the virulence of the *lytST* and *dltAD* genes of *S. mutans* in the development and severity

of carious lesions (in a rodent model of dental caries) and virulence in a systemic infection model (*Galleria mellonella*).

MATERIAL AND METHODS

Bacterial Strains

Streptococcus mutans UA159, serotype *c* (ATCC 700610), a proven cariogenic organism, was the parental strain used in this study. Mutant strain derivatives of UA159 were created carrying deletions in *lytS*, *lytT*, *dltA* and *dltD*. The deletion strains for *lytS*, *lytT* [25], *dltA,* and *dltD* [26] came from the collection of mutant strains created in the Quivey laboratory (kindly provided by Dr. Robert G. Quivey Jr, Center for Oral Biology, University of Rochester, Rochester, NY). In addition, a strain defective in *gtfB* was used as a control, because it displays impaired biofilm formation *in vitro* [11,27], and reduced pathogenicity *in vivo* [28]. The *gtfB* mutant was kindly provided by Dr. Robert A. Burne (Department of Oral Biology, University of Florida, Gainesville, FL). The strains were stored at –80ºC in tryptic soy broth containing 20% glycerol and were plated on blood agar plates or BHI agar plates with erythromycin (5 µg / mL, Sigma) before use in the following assays.

Rat model of dental caries

The animal experiment was reviewed and approved by the Ethical Committee on Animal Research at the University of Campinas (CEUA - Ethics Committee on Animal Use/UNICAMP, Campinas, SP, Brazil, process number 4463-1 / 2017) (ANEXO B) and was performed according to methods previously described [29,30]. An overview of the experimental design is shown in Figure 1. A total of 56 SPF (Specific Pathogen Free) female Wistar rats, aged 19 days were provided by CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, Campinas, SP, Brazil). From the arrival of the animals until the end of the experiment, the animals were kept in a room of the vivarium for research using Genetically Modified Organisms (GMOs), authorized and approved by the GMO Biosafety Committee from FOP/UNICAMP (A001-2017) (ANEXO C).

On the day after the arrival of the animals, the animals were randomized to form four groups composed of 14 animals, using ear tags. The four groups were color-coded, so the investigators handling the animals were blinded to the strain that would be used for infection. The black group was infected with the parental strain, UA159 (positive control strain). The red group was infected with Δ*gtfB* (a reduced-caries-causing control to which the caries-causing potential of the tested strains was compared). The blue and green groups were infected with Δ*lytS* and Δ*dltD*, respectively.

Each animal was weighed (COMTEC 426-1070) and the data recorded. Initial screening was performed to verify that the animals were not colonized with mutans streptococci. A sterile cotton bud (Labor Import), wetted in saline solution (0.89% NaCl), was used to swab the rat mouths. Each oral swab was struck on a Mitis Salivarius agar plate without antibiotic (MSA) and on a Mitis Salivarius agar plate containing 0.2 U of bacitracin per ml (MSB). The plates were incubated (37°C, 95% (v/v) air/ 5% (v/v) CO_2, 48h) and the morphologies of colonies were visually evaluated to ensure that the animals were free of mutans streptococci colonization.

Biofilm and inocula preparation for caries animal study. The four strains (parental strain UA159 and deletion strains *ΔgtfB*, *ΔlytS,* and *ΔdltD*) were grown in biofilm cultures on glass rods in the presence of sucrose. It has been shown that using biofilm cultures as the inoculum in the rat mouth enhances their ability to colonize tooth surfaces [31,32]. On day one of biofilm growth, 50 µL of each strain was transferred to a tube containing TY (tryptone plus yeast extract, Difco) and 2% sucrose (Dinamica® / 1894-1), antibiotic was added to media for cultures of the deletion strains. After incubation at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere for 24h (day two of biofilm growth), each rod was transferred to new tubes with fresh media containing 2% sucrose. These cultures were incubated under the same conditions described above, and the rods transferred to fresh media again on day three of biofilm growth. On day four of biofilm growth, biofilms were scraped off the rods and transferred to a new tube containing fresh media, then incubated as above. After 18h, these cultures were plated on MSB agar plates and incubated for 48h, after which time the plates were stored at 4°C to be utilized as inocula to infect the animals.

On the day before the arrival of the animals, starter cultures were prepared by inoculating 10 mL of TY liquid medium containing 1% glucose (Synth) with colonies

from the plates described above. Two tubes were prepared for each strain and incubated at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere. After 18 h, the starter cultures were diluted in TY liquid medium containing 1% glucose (1:20 dilution). The diluted cultures were allowed to grow to an $O.D._{540nm}$ ~ 0.5. Each culture was then transferred to a 50 mL tube containing 14 swabs. These tubes were maintained on ice until shortly before inoculating the animals' mouths, at which time the cultures were warmed to room temperature.

Infection and maintenance of animals. Rats were infected with the cultures described above by oral swabbing four times, on four consecutive days (as outlined in Figure 1). After these four infection procedures, a confirmatory screening was performed to verify the presence of the *S. mutans* strains in the mouth of each rat, similar to the procedure used in the initial screening. Immediately after the confirmatory screening, the animals were transferred to suspended cages (two animals per cage), where they remained for five weeks until sacrificed.

After the first infection, the rats' diet was replaced with Diet 2000 (containing 56% sucrose [Table S1; 33]). The animals were given sterile, distilled, deionized, water containing 5% sucrose, ad libitum. The ad libitum cariogenic diet used during the five-week experimental period is an established method for allowing the development of carious lesions on the dental surfaces [9]. The animals were weighed weekly (Figure S1). In the second and fourth weeks, the vitamin Vitagold Potenciado (Fabiani Saúde Animal, São Paulo, Brazil) was added to the drinking water (20 mL per 10 L of water) to prevent hair loss.

Recovery of jaws from animals. The animals were euthanized by anesthesia using Ketamine (Dopalen 1000mg / 10 mL - Ceva ®) (300 mg / kg) and Xylazine (Anasedan 2000mg / 10 mL - Ceva ®) (30 mg / kg), followed by decapitation.

The lower left jaw of each animal was aseptically dissected and was suspended in 5 mL of sterile 0.89% NaCl solution. The jaws were sonicated twice, with a 10-s interval between probe sonication for 30 s at 7 W (Vibracell, Sonics and Material Inc., Newtown, CT). Aliquots of each biofilm suspension were used for 10-fold serial dilutions. The dilutions 1:10, 1:100 and 1:1000 and undiluted suspension were seeded on blood agar medium (for total cultured microbiota) and MSB agar medium (for *S. mutans*), and the plates were incubated for 48 h at 37°C in a 95% (v/v) air/ 5% (v/v)

CO_2 atmosphere. The blood agar plates were incubated for another 24h at 37°C. Colonies were counted and used to calculate CFU in 5 mL of biofilm suspension (CFU/biofilm). All jaws were stored at -20°C until manual dissection for caries scoring.

Caries Scoring. The teeth were prepared for caries scoring according to Larson's modification of Keyes' system [34]. One calibrated examiner performed the caries score of the codified jaws. The smooth surface caries scoring (buccal, morsal, proximal, lingual, and palatine) was done using a stereoscopic Zeiss magnifying lens (Stemi SV 6).

Before performing caries scoring on sulcal surfaces, the samples were prepared as follows: The jaws were painted with transparent nail polish to strengthen the structure and left to dry for 24 h. Murexide (SIGMA M-2628) dye solution (0.24 mg/mL) was used to stain the jaw and left to dry for 18 h. A sagittal cut was made in the jaws with carbide disc coupled to a micromotor (mc52 - dent) to allow for better visualization of the sulcal surface. Caries scoring was performed on the sulcal surfaces using a stereoscopic Zeiss magnifying lens (Stemi SV 6).

Infection study in the invertebrate model Galleria mellonella

Five deletion strains (Δ*gtfB,* Δ*lytT,* Δ*lytS,* Δ*dltA,* and Δ*dltD*) and the parental strain (UA159) of *S. mutans* were used to examine pathogenicity in the *Galleria mellonella* model of infection. The strains were cultured as described in ***Bacterial Strains***. Starter cultures of each of the six strains were prepared in 10 mL of TY liquid medium containing 1% glucose and incubated at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere. After 18 h, the cultures were diluted in TY liquid medium containing 1% glucose (1:20 dilution) and grown to an $O.D._{540nm}$ ~ 0.5.

Cells were collected by centrifugation (6,500 x *g* / 4°C / 15min, Hettich Zentrifugen, rutin 420R) and pellets were washed twice with 3 mL of sterile 0.89% NaCl solution. Cell pellets were resuspended in 3 mL 0.89% NaCl. Briefly, a 100 µL aliquot of each strain suspension was transferred to a tube containing 900 µL of 0.89% NaCl solution to perform 10-fold serial dilutions. The 10^{-7} dilution was plated on blood agar (in triplicate), and the plates were incubated at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere. After 48h, the colonies were counted and used for the calculation of CFU. A heat-killed control for each strain was incubated for 40 min at 85 °C.

The systemic infection of *G. mellonella* was performed as described previously [35-37]. The larvae were stored at 32°C, in the dark, in a greenhouse (SOLAB BODSL-200/364, Solab, Piracicaba, Brazil) until the experiment. For each bacterial strain tested, a group of 10 larvae, varying from 200 to 300 mg and without signs of melanization, were selected for inoculation with the six strains of *S. mutans* (parental UA159, Δ*gtfB*, Δ*lytT*, Δ*lytS*, Δ*dltA,* and Δ*dltD*) and the heat-inactivated control strains (all infections were performed in triplicate).

Five µL of *S. mutans* from each strain containing 1-1.5 × 10^8 CFU was injected *intra*-hemocoel via the last left proleg using a Hamilton syringe (Hamilton, Reno NV) [38]. After injection, the larvae were placed in Petri dishes for incubation at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere. The appearance of melanization and larval survival were recorded three times per day according to the *G. mellonella* Health Index Scoring System [39]. The larvae were classified as dead when they displayed no movement in response to touch.

Tolerance to oxidative stress by hydrogen peroxide (H_2O_2)

Planktonic cultures and biofilms of the six strains of *S. mutans* (parental UA159, Δ*gtfB*, Δ*lytT*, Δ*lytS*, Δ*dltA,* and Δ*dltD*) were subjected to an H_2O_2 challenge, as oxidative stress is part of the innate immune system of *G. mellonella* [38,40]. The planktonic cultures were grown as described above for the infection of larvae. Cultures were exposed to 0.2% H_2O_2 for 0, 5, 15, 30 and 45 minutes. At the end of the exposure time, aliquots (100 µL) were used for 10-fold serial dilutions that were plated on blood agar plates.

The biofilm cultures were grown on vertically placed hydroxyapatite disks (1.25 cm diameter, Chromatography products Clarkson, Inc., South Williamsport, PA), coated with filter-sterilized clarified human whole saliva (sHA). The saliva and pellicle preparation were performed as described before [8]. The saliva collection and use were approved by the Institutional Ethics Committee on Human Research at UNESP (CAAE: 31717914.3.0000.5416) (ANEXO D). Biofilm formation was performed as described by Castillo Pedraza at al. [8]. Briefly, the strains were grown in tryptone-yeast extract broth (TY, 2.5% tryptone, 1.5% yeast extract) containing 1% glucose at 37°C in a 95% (v/v)

air/ 5% (v/v) CO_2 atmosphere until reaching late exponential growth phase (OD_{540nm} ~1.0).

Next, each strain was inoculated individually (10^6 CFU mL^{-1}) in 2.8 mL of TY with 0.1% sucrose and 25% saliva and incubated at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere. The beginning of this incubation was considered day 0 (biofilm age 0 h). At 19 h, the culture medium was replaced by transferring the custom-made disk holder with nascent biofilms to wells containing fresh medium (TY + 0.1% sucrose and 25% saliva) using forceps. After 29 h of biofilm growth, biofilms were transferred to TY containing 0.5% sucrose, 1% starch, and 25% saliva to increase the amount of carbohydrate serving as substrates for microbial metabolism and matrix accumulation. The culture medium was then changed twice daily (8 am and 6 pm) until the end of the experimental period. Thus, the biofilms were kept immersed in 0.1% sucrose/25% saliva for 10 h, and in 0.5% sucrose/1% starch/25% saliva for 14 h. Biofilms were grown up to 67 h, and during all ages were incubated with the same initial environment as 0 h (at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere).

At 67 h biofilms were removed from the culture medium, rinsed (three times) with 0.89% NaCl solution, and transferred to wells of a 24-well plate containing 0.2% H_2O_2 and incubated for 15, 30 and 45 min. At time 0 min, biofilms were incubated without H_2O_2. At the end of the exposure time, the disks were transferred to tubes containing 2 mL 0.89% NaCl solution and sonicated for 10 min (in an ultrasonic bath) to harvest biofilms from the disks. The disks were scraped with a sterile spatula to remove residual biofilm. The biofilms were homogenized with a probe for 30 s at 7 W (Sonicator model Q125, QSonica). From these biofilm suspensions, aliquots (100 µL) were used for 10-fold serial dilutions that were plated on blood agar plates.

For both planktonic and biofilm cultures, the plates were incubated for 48 h at 37°C in a 95% (v/v) air/ 5% (v/v) CO_2 atmosphere and the colonies were counted to obtain CFU/mL (planktonic cultures) or CFU/biofilm (biofilms). The population recovered at 0 min was set at 100% per strain for planktonic and biofilm cultures.

Statistical analyses

The virulence potential of the different deletion strains and the parental strain of *S. mutans* were analyzed using Prism 7 software (GraphPad Software, Inc.). The

microbial population data (total culturable microbiota, *S. mutans*, and percentage of *S. mutans*) recovered from rats were evaluated with analysis of variance (ANOVA) one-way followed by Tukey's test (α = 0.05). The development of carious lesions was evaluated by two-way ANOVA, followed by the Tukey's test (α = 0.05) using as factors "*S. mutans* strain" and "type of caries lesion on smooth and sulcal surfaces". The amount of CFU inoculated per strains of *S. mutans* in *G. mellonella* larvae was analyzed by one-way ANOVA, followed by the Tukey's test (α = 0.05). The virulence potential of *S. mutans* in the invertebrate model was assessed using the Kaplan-Meier survival curve and estimates of survival differences were compared using the log-rank test. Oxidative stress tolerance (H_2O_2) was evaluated using ANOVA two-way followed by Tukey's test (α = 0.05) using as factors "*S. mutans* strain" and "exposure time" for both planktonic cultures and biofilms.

RESULTS

Loss of lytS and dltD reduced caries formation in a rat model

Total cultivable flora recovered from rats used in a model for caries formation revealed that rats infected with the parental strain UA159 and the Δ*gtfB* and Δ*lytS* strains, did not show significant differences between them, but the Δ*dltD* strain had fewer CFU/mL, compared to the parental strain UA159 ($p = 0.0224$, one-way ANOVA, followed by the Tukey's test; Figure 2(a)). Infection with the Δ*gtfB* strain, our reduced caries control, resulted in a lower *S. mutans* population, compared to the other strains tested (parental strain UA159 and Δ*lytS* strain) except for the Δ*dltD* strain (no significant difference between Δ*gtfB* and Δ*dltD*; Figure 2(b)). The proportion of *S. mutans* in the total microbiota was higher for the parental strain UA159 compared to all deletion strains tested ($p < 0.0001$, one-way ANOVA, followed by the Tukey's test; Figure 2(c)). Moreover, the percentage of *S. mutans* for Δ*gtfB* was lower than for Δ*lytS* ($p = 0.0027$), and there was no difference between Δ*gtfB* and Δ*dltD* strains ($p = 0.2036$). Therefore, there was a successful implantation of *S. mutans* in the oral microbiota of all rats evaluated.

All strains tested caused dental caries on smooth and sulcal surfaces (Figure 3). There were significant changes in smooth carious lesion scores for rats infected with the deletion strains, compared to UA159 (Figure 3(a)); however, there was not a

significant difference among the mutant strains. Animals infected with the *ΔlytS* strain exhibited a greater number of smooth surface carious lesions on enamel (E) and dentin (Ds and Dm) than rats infected with *ΔgtfB* and *ΔdltD* (Figure 3(a)). None of the mutant strains tested caused severe dentinal caries (Dx) on smooth and sulcal surfaces, though the rats infected with the Δ*lytS* strain displayed a significantly higher number of slight dentinal caries (Ds) on smooth surfaces than the Δ*dltD* strain (Figure 3(a)). These data suggest that *lytS* and *dltD* could play an important role in the development of caries on smooth surfaces. This observation has already been recorded for smooth surface lesions in animals infected with a Δ*gtfB* strain [28].

Sulcal carious lesions were reduced in rats infected with mutant strains, compared to infection with UA159. Moreover, there was no difference between the mutant strains in severity, but their scores were lower than those from UA159 infected animals (Figure 3(b)). Importantly, animals infected with the deletion strains also did not display Dx lesions on sulcal surfaces, suggesting that the products of the *lytS*, *dltD* and *gtfB* genes could also play an essential role in the development of caries on sulcal surfaces. Further, the loss of *lytS*, *dltD* and *gtfB* not only influenced the development of cariogenic biofilms *in vitro* [8] but also *in vivo*.

Strains carrying gene deletions gtfB, lytT, lytS, dltA, and dltD prevented the killing of G. mellonella larvae

The deletion strains were then tested in an *in vivo* model of infection, *Galleria mellonella*. Using similar inocula (Figure S2), all the deletion strains were able to survive longer in the larvae than the parent strain, UA159, which killed all larvae after 67 hours of infection (Figure 4; Table S2). In addition, only 7% of the larvae inoculated with Δ*gtfB* survived at 72 hours, demonstrating higher systemic virulence potential of this strain when compared to the other deletion strains. Specifically, the larvae inoculated with Δ*lytT* (16.6%), Δ*lytS*, (40%), Δ*dltA* (40%) and Δ*dltD* (50%) showed a higher percentage of survival at 72 hours after inoculation. Thus, the Δ*dltD* strain was significantly less able to cause larvae killing, compared to the other deletion strains tested (Figure 4). These results indicate that the products of the genes examined in this study may play a role in influencing how *S. mutans* thrives in systemic infections.

S. mutans strains tolerance to hydrogen peroxide

To further examine the mechanism by which the gene deletion strains were able to survive in the *G. mellonella* larvae, the ability of the strains, grown in planktonic or biofilm cultures, to survive exposure to H_2O_2 was determined. In planktonic cultures, the parental strain exhibited the greatest ability to survive a peroxide challenge following 45 minutes of exposure. There was a gradual decrease in the survival percentage of *S. mutans* in planktonic cultures (Figure 5(a)) and biofilms (Figure 5(b)) of Δ*lytT*, Δ*lytS*, Δ*dltA*, and Δ*dltD* strains after exposure to H_2O_2, except for the planktonic culture of Δ*lytT* that increased its population when it passed from 30 to 45 min. However, the Δ*gtfB* strain was significantly better able to withstand a peroxide challenge when grown in a biofilm culture. This finding suggests that a decreased amount of insoluble exopolysaccharides found in the extracellular matrix of Δ*gtfB* biofilm cultures [8] did not facilitate H_2O_2 killing, and other factor(s) may be playing a role (*e.g.*, cell membrane composition and turnover). The ability of the Δ*gtfB* strain to persist in biofilm cultures exposed to H_2O_2 is borne out in the results observed in our in vivo *G. mellonella* infection model (Figure 4).

DISCUSSION

The present study demonstrated that the *lytST* and *dltAD* genes affect *S. mutans* virulence in a rodent model of caries and a systemic infection assay. All rats infected with the *S. mutans* parental strain UA159 and deletion strains developed carious lesions. Greater numbers of enamel and dentinal lesions were measured in teeth of animals infected with the parental UA159 strain on the smooth and sulcal surfaces of the animals' teeth. However, in the animals infected with the deletion strains Δ*gtfB,* Δ*lytS* and Δ*dltD*, caries lesions were less severe; moreover, these deletion strains did not cause severe dentinal caries. Thus, the absence of the products encoded by *gtfB, lytS* and *dltD* resulted in decreased cariogenicity of *S. mutans*. In addition, loss of the *gtfB, lytS, lytT, dltA*, and *dltD* gene products prevented the killing of *G. mellonella* larvae after systemic infection with *S. mutans* (compared to the UA159 parental strain), indicating that these five gene products may also influence systemic infections, by reducing the virulence of the organism. This reduction in systemic infection may be in part due to the absence of these genes made the deletion strains (except Δ*gtfB*) more

susceptible to oxidative stress, which is part of the innate immune system of *G. mellonella*.

The two in vivo models were selected with distinct purposes; the rodent model was to assess the cariogenic potential of strains with deletion of specific genes, and the invertebrate model as an incipient model to evaluate whether the deleted genes could impair systemic infection potential of *S. mutans.* Specifically, because the deletion strains caused fewer caries lesions with lower severity (vs. the parental strain), the specific genes (and their products) could be targets for directed preventive/therapeutic approaches. Thus, if such approaches would be implemented, there was no information whether targeting these genes would interfere with *S. mutans* behavior in case of bacteremia (e.g., once it goes to the host bloodstream during oral hygiene procedures like flossing). There was no information whether the attenuated virulence phenotype to cause caries would also present an attenuated phenotype in a systemic infection. Therefore, a simple systemic model (larvae) was selected to verify how the deletion strains would affect *S. mutans* virulence.

In the caries model, the microbial population recovered from rats showed a higher proportion of *S. mutans* in the total microbiota for UA159 versus deletion strains (15-fold for Δ*gtfB,* 3-fold for Δ*lytS*, and 6-fold for Δ*dltD*). Recently, a library of UA159 transposon mutants was inoculated in mice to identify genes that are important for colonization and survival in the oral cavity; after 3 weeks, the mutant strains *gtfB, dltC*, *dltD,* and *lytT* presented lower recovery, while *dltA*, *dltB,* and *lytS* were recovered at similar rate as UA159, but no caries status was reported [41]. Here, the deletion strains Δ*dltA* and Δ*dltD* growth in TY+1% glucose was slower compared to the parental strain UA159 and the deletion strains Δ*gtfB,* Δ*lytS*, and Δ*lytT*. These two strains took 40 to 60 min more to reach mid-exponential ($O.D._{540nm}$ ~ 0.5) and late exponential growth phase ($O.D._{540nm}$ ~ 1.0). Thus, only Δ*dltA* and Δ*dltD* presented a slow-growth defect that may explain in part the outcome for lower colonization in the rats' teeth and fewer and less severe carious lesions observed. Initial work with Δ*lytT* (Δ*lytR*) grown in Brain Heart Infusion broth demonstrated a defect in cell division leading to longer chains because of defects in autolysin activity but without changes in planktonic growth rate or biofilm formation [25]. Later, in a comprehensive study of a genomic collection of gene deletion mutants, the strains were categorized into several growth traits [26]. Both Δ*dltA* and Δ*dltD* had poor acid survival in planktonic cultures and single-species biofilm

formation deficiencies on polystyrene surfaces in the presence of sucrose or glucose [26]. The deficiency in biofilm formation on hydroxyapatite was not observed in our *in vitro* study, which used TY culture media with saliva and sucrose alternated with sucrose plus starch [8]. Furthermore, the pH of spent culture media did not differ between strains grown in single-species biofilms, indicating that all strains grew as pH of culture media dropped from 7.0 to ~4.5 [8]. Nevertheless, the slower growth and lower acid tolerance of Δ*dltA* and Δ*dltD* may contribute to the lower percentage of *S. mutans* recovered and fewer (and less severe) caries lesions.

In the current study, for smooth surfaces, UA159 yielded ≥50% more lesions than the deletion strains on enamel, while it caused the highest amount of caries lesions in dentin (≥80% more cavities). For sulcal surfaces, UA159 yielded ≅30% more lesions than the deletion strains on enamel, while UA159 also caused a higher amount of caries lesions in dentin (being ≥60% more severe than the deletion strains). *S. mutans* possesses several virulence factors associated with cariogenicity such as adhesion capacity, acidogenicity, acid tolerance, production of bacteriocins (mutacins) and biofilm formation [10,42]. However, the ability to synthesize water-insoluble exopolysaccharides is a trait that makes *S. mutans* a significant pathogen of dental caries [2]. The synthesis of exopolysaccharides depends on the expression of Gtfs by this microorganism in the presence of dietary carbohydrates [9]. Lack of working *gtfB* was related to low levels of carious lesions on the smooth surfaces of rat teeth [28,43]. Thus, it was expected that the *ΔgtfB* strain would cause less carious lesions on the surfaces of the teeth in the present study.

The infection by Δ*lytS* caused fewer caries lesions in enamel and dentin of rats than the parental strain UA159. Thus, the interruption of the two-component system *lytST* affected *S. mutans* cariogenicity. The *lytST* complex is essential for *lrgAB* expression in *S. mutans*, which participates in oxidative stress tolerance, biofilm build-up and regulation of autolysis [44,45]. The eDNA released by autolysis interacts with GtfB and LTA and affects the production of exopolysaccharides, colonization by *S. mutans*, and construction of the extracellular matrix [8,12,17]. Previously, our group described an increased amount of eDNA in the matrix of *in vitro* biofilms formed by Δ*lytS* and Δ*lytT* [vs. the parental strain; 8]. We proposed that the loss of these genes was affecting *S. mutans* cell metabolism by increasing cell wall turnover, thereby increasing the secretion of microvesicles containing eDNA [17], or triggering additional

pathways that culminate in the augmented release of DNA into the matrix [8]. The induction of additional pathways could explain the decreased cariogenicity of Δ*lytS in vivo* observed in this study.

In addition, the *ΔdltD* strain also showed a lower number of caries lesions in enamel and dentin of rats (versus the parental strain UA159), suggesting that the product of the *dltD* gene could also be involved in the cariogenicity of *S. mutans*. This finding was expected since the *dltABCD* operon genes are required for the addition of D-alanine residues during LTA synthesis [19]; these residues affect bacterial adhesion to the surface and formation of biofilms [22,23,46,47]. Also, a previous *in vivo* study induced the expression of the *dltABCD* operon and demonstrated a higher occurrence of caries [48]. Furthermore, the *S. mutans* proteins encoded by the *dltA, dltC* and *dltD* genes were abundant during the early stages of cariogenic biofilm formation and were related to the amounts of intracellular polysaccharides [15,23]. Therefore, the loss of genes related to the synthesis of exopolysaccharides, eDNA, and LTA metabolism impairs cariogenicity of *S. mutans*.

In an earlier *in vitro* study, it was expected that the deletion of *lytS* and *lytT* would decrease the amount of eDNA, and deletion of *dltA* and *dltD* would reduce the quantity of LTA in the matrix (*versus* the parental strain). However, the data showed more eDNA and LTA in biofilms with the respective deletion strains and that the 3D structure was distinct [8]. Based on these data, it was expected that the strains yielding more eDNA and LTA in the matrix would cause more caries lesions; but this was not the outcome in the rodent model of dental caries here. An explanation is that the deletion of *lytST* and *dltAD* alters *S. mutans* physiology to form biofilms with distinct quantities of extracellular matrix components *in vitro* [8]. These traits could be because of alterations in the cell wall turnover and composition (and charge), as suggested previously [13,44,45,49,50]. The cell wall works as an ‘anchor’ for matrix components to connect to cells surface and mediates cell-cell binding, cell-matrix, cell-matrix-dental surface. Hence, alterations in this ‘anchor’ could lead to a weaker, smaller (with fewer cells connected to each other and to the matrix; that is, lower percentage of *S. mutans* recovered from rats’ dental biofilms) and more ‘porous’ 3D structure that would retain less acid inside the biofilm and at the interface biofilm/dental surface, causing fewer caries lesions. Thus, a more ‘porous’ 3D structure might be related to the distinct patterns of the distribution of bacteria and exopolysaccharides observed in the *in vitro*

biofilms (vs. parental strain UA159), represented as percentage coverage per area from the interface substratum/biofilm (hydroxyapatite disk) to the top (outer layer) of each biofilm [8].

Oral bacteria can enter the bloodstream, and usually, bacteremia can be rapidly eliminated by the immune system. However, it has been shown that if *S. mutans* persists in the bloodstream, it can colonize the endothelial cells of the coronary arteries [36]; the cerebral arterial wall [51], the renal arterial wall [52], and even reach the hepatic veins in the liver [53]. *G. mellonella* has a pathogenic defense mechanism similar to the innate immune system of mammals [54], and their hemolymph is considered an analog to blood in vertebrates. This invertebrate model was used to analyze *S. mutans* pathogenesis in systemic diseases such as bacterial endocarditis [35-37]. Here, the deletion of the *gtfB, lytS, lytT, dltA*, and *dltD* genes caused lower and slower larval mortality rates. However, the Δ*gtfB* strain had a higher capacity to kill more larvae when compared to the other deletion strains. The larvae defense mechanisms include a plasmacyte-mediated melanocyte process [38], and the production of reactive oxygen species (ROS), enzymes, and antimicrobial peptides [40]. But, as *G. mellonella* is a limited, preliminary systemic model, further studies will need to be performed to address the contribution of these genes to *S. mutans* systemic virulence. In addition, the mechanism by which *S. mutans* reacts to the stress caused by the immune system of *G. mellonella* is still to be elucidated.

Nevertheless, *S. mutans* is susceptible to oxidative stress [55]. Here, the Δ*lytS,* Δ*lytT,* Δ*dltA* and Δ*dltD* strains revealed lower tolerance to H_2O_2, making these genes important for providing resistance to oxidative stress. The Δ*gtfB* strain showed greater survival to H_2O_2 exposure than the other deletion strains, possibly due to the presence of *lytST* and *dltAD* that could together alleviate the deleterious effects of hydrogen peroxide. The lack of a protective matrix in *ΔgtfB* could render this strain more susceptible to oxidative challenge, but the data showed an opposite outcome. Thus, we hypothesize that the absence of a working *gtfB* gene changes the cell wall/membrane making *S. mutans* cells more tolerant to oxidative stress when grown in a biofilm. Further research is needed to pinpoint the exact mechanism for Δ*gtfB* higher tolerance to H_2O_2 and explain why it killed more larvae than the other four deletion strains tested. For example, transcriptomic and/or proteomic analyses of Δ*gtfB* challenged with H_2O_2 cold indicate the mechanisms involved in the observed tolerance.

Nevertheless, previous gene expression analysis indicated that *ΔgtfB* might present a slower turnover in the cell membrane in mixed-species biofilms [13, used the same strain *ΔgtfB* used here], corroborating with earlier work using a *ΔgtfBC* strain [56].

Moreover, bacterial adaptation to changing environmental conditions is often accomplished by TCSs that modulate gene expression in response to many stimuli [57]. A previous study showed that the Cid/Lrg systems interact together to favor the colonization of *S. mutans* against environmental stresses [58]. The expression of *lrgAB* is dependent on the *lytST* genes and confers resistance to oxidative stress [44,45]. LytST (*lytS*: sensor kinase; *lytT*: response regulator) is required to activate the expression of *lrgAB*, which are part of the *S. mutans* arsenal that controls biofilm formation and autolysis [45]. The *S. mutans lrgAB/lytST* deletion strains exhibited a decreased growth rate in the presence of several ROS [44,45]. Before, the expression pattern was distinct for *lytS* and *lytT*. The expression of *lytS* was influenced by pH and abundance of carbohydrates, while *lytT* was expressed in very low magnitudes at distinct developmental stages for both UA159 and Δ*gtfB* in mixed-species biofilms, regardless of environmental pH or nutrient availability [13]. The similarity between both types of biofilms indicates that *lytT* may interfere with multiple metabolic pathways simultaneously.

Furthermore, as the Δ*gtfB* biofilm aged, the expression of *lytS* decreased, being inversely proportional to the increase of expression recorded for *lrgAB*, which was not observed for UA159 biofilms. Even though LytST controls the expression of *lrgAB,* a Δ*lrgAB* mutant presented a distinct transcriptomic profile from the Δ*lytS* mutant [45], suggesting that changes in gene expression by the mutation of *lrgAB* could be independent of LytST [59]. Further comparison of transcriptomics and proteomics of a Δ*lrgAB* mutant (vs. the parental strain) subjected to distinct oxidative, heat and antimicrobial stresses indicated 'that adaptation to a new environment may require radical proteome turnover or metabolic remodeling' [50]. Among the pathways (and resulting products) were several genes/proteins involved in cell wall biogenesis affecting cell wall turnover and stress tolerance (e.g., GtfC, AtlA, DltC) [50]. In addition, alteration in the cell wall of dlt mutants may also occur.

The deletion strains Δ*dltA* and Δ*dltD* exhibited the same behaviors as the Δ*lytS* and Δ*lytT* when inoculated in *G. mellonella* larvae. Thus, these genes could also be contributing to the virulence of *S. mutans* in systemic diseases and cooperation for the

tolerance to ROS products. Moreover, the expression of the *dlt* genes was related to the resistance of *S. mutans* biofilms against gentamicin, a drug used to treat endocarditis [49]. The *ΔdltA* showed a more negatively charged surface than the parental strain, reducing the tolerance of the *dltA* mutant biofilms towards the positively charged gentamicin [49]. Furthermore, it was shown that *dltABCD* affects the susceptibility of *S. mutans* to cationic antimicrobial peptides (innate immune factors in humans) [60]. It has been shown that a D-Ala analog can block the activity of DltA and synergize with the peptidoglycan synthesis inhibitor of vancomycin, leading to growth inhibition [61]. Additionally, a small molecule was identified as a LtaS enzyme inhibitor [62] that inhibit the growth of several medically critical Gram-positive bacteria [62,63]. Our preliminary work with this small molecule has shown to inhibit *S. mutans* growth in planktonic culture and biofilms (Castillo Pedraza *at al*., unpublished data).

Therefore, the higher survival of larvae infected with Δ*lytS,* Δ*lytT,* Δ*dltA,* and Δ*dltD* may be related to the strains' lower tolerance to oxidative stress after exposure to H_2O_2, and host antimicrobial peptides [60]. Thus, it is possible that changes on cell wall/membrane in the deletion mutant tested here could explain some of the distinct and even unexpected outcomes, and thus, future research on cell wall/membrane composition and structure is warranted.

In conclusion, besides the roles that the *lytST* and *dltAD* genes play in the development and composition of *in vitro* biofilms [8], the results from this study demonstrate that they are also factors in the virulence of *S. mutans* in the development of dental caries and systemic infection. Inhibition of these genes and their products with specific therapies would help decrease the virulence of *S. mutans* to prevent the deleterious effects of demineralization and reduce microorganism survival in systemic infections.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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REFERENCES

1. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986;50(4):353–380.
2. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res. 2013;92(12):1065–1073.
3. Nakano K, Fujita K, Nishimura K, et al. Contribution of biofilm regulatory protein A of *Streptococcus mutans*, to systemic virulence. Microbes Infect. 2005;7 (11–12):1246–1255.
4. Nakano K, Ooshima T. Serotype classification of *Streptococcus mutans* and its detection outside the oral cavity. Future Microbiol. 2009;4:891–902.
5. Nakano K, Nemoto H, Nomura R, et al. Detection of oral bacteria in cardiovascular specimens. Oral Microbiol Immunol. 2009;24(1):64–68.
6. Branda SS, Vik S, Friedman L, et al. Biofilms: the matrix revisited. Trends Microbiol. 2005;13(1):20–26.
7. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8(9):623–633.

8. Castillo Pedraza MC, Novais TF, Faustoferri RC, et al. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of cariogenic biofilms. Biofouling. 2017;33:722–740.
9. Bowen WH, Koo H. Biology of *Streptococcus mutans* derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45(1):69–86.
10. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011;90(3):294–303.
11. Xiao J, Klein MI, Delahunty CM, et al. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog. 2012;8(4):e1002623.
12. Klein MI, Hwang G, Santos PHS, et al. *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol. 2015;5:10.
13. Florez Salamanca EJ, Klein MI. Extracellular matrix influence in *Streptococcus mutans* gene expression in a cariogenic biofilm. Mol Oral Microbiol. 2018;1–13.
14. Klein MI, DeBaz L, Agidi S, et al. Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One. 2010;5(10):e13478.
15. Klein MI, Xiao J, Lu B, et al. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. PLoS One. 2012;7(9):e45795.
16. Klein MI, Duarte S, Xiao J, et al. Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilms development. Appl Environ Microbiol. 2009;75(3):837–841.
17. Liao S, Klein MI, Heim KP, et al. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. J Bacteriol. 2014;196(13):2355–2366.
18. Ellwood DC, Tempest DW. Effects of environment on bacterial wall content and composition. Adv Microb Physiol. 1972;7:83–116.
19. Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev. 2003;67:686–723.

20. Kuramitsu HK, Wondrack L, McGuinness M. Interaction of *Streptococcus mutans* glucosyltransferases with teichoic acids. Infect Immun. 1980;29(2):376–382.
21. Rölla G, Oppermann RV, Bowen WH, et al. High amounts of lipoteichoic acid in sucrose induced plaque in vivo. Caries Res. 1980;14(4):235–238.
22. Gross M, Cramton SE, Gotz F, et al. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun. 2001;69:3423–3426.
23. Spatafora GA, Sheets M, June R, et al. Regulated expression of the *Streptococcus mutans* dlt genes correlates with intracellular polysaccharide accumulation. J Bacteriol. 1999;181(8):2363–2372.
24. Reichmann NT, Gründling A. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol Lett. 2011;319:97–105.
25. Chatfield CH, Koo H, Quivey RG Jr. The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated. Microbiology. 2005;151(2):625–631.
26. Quivey RG, Grayhack EJ, Faustoferri RC, et al. Functional profiling in *Streptococcus mutans*: construction and examination of a genomic collection of gene deletion mutants. Mol Oral Microbiol. 2015;30 (6):474–495.
27. Koo H, Xiao J, Klein MI, et al. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. J Bacteriol. 2010;192 (12):3024–3032.
28. Yamashita Y, Bowen WH, Burne RA, et al. Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model. Infect Immun. 1993;61:3811–3817.
29. Bowen WH, Pearson SK, Young DA. The effect of desalivation on coronal and root surface caries in rats. J Dent Res. 1988;67(1):21–23.
30. Bowen WH, Schilling K, Giertsen E, et al. Role of a cell surface associated protein in adherence and dental caries. Infect Immun. 1991;59(12):4606–4609.
31. Ma Y, Rutherford GC, Curran TM, et al. Membrane locus and pH sensitivity of paraben inhibition of alkali production by oral streptococci. Oral Microbiol Immunol. 1999;14(4):244–249.

32. Duarte S, Rosalen PL, Hayacibara MF, et al. The influence of a novel propolis on mutans streptococci biofilms and caries development in rats. Arch Oral Biol. 2006;51(1):15–22.
33. König KG, Larson RH, Guggenheim B. A strain-specific eating pattern as a factor limiting the transmissibility of caries activity in rats. Arch Oral Biol. 1969;14(1):91–103.
34. Larson RM. Merits and modifications of scoring rat dental caries by Keyes' method. Symposium on Animal Models in Cariology; Washington, DC; 1981. p 203.
35. Abranches J, Miller JH, Martinez AR, et al. The collagen-binding protein cnm is required for *Streptococcus mutans* adherence to and intracellular invasion of human coronary artery endothelial cells. Infect Immun. 2011;79:2277–2284.
36. Aviles-Reyes A, Miller JH, Simpson-Haidaris PJ, et al. Cnm is a major virulence factor of invasive *Streptococcus mutans* and part of a conserved threegene locus. Mol Oral Microbiol. 2014;29:11–23.
37. Freires IA, Avilés-Reyes A, Kitten T, et al. Heterologous expression of *Streptococcus mutans* Cnm in Lactococcus lactis promotes intracellular invasion, adhesion to human cardiac tissues and virulence. Virulence. 2017;8(1):18–29.
38. Cotter G, Doyle S, Kavanagh K. Development of an insect model for the in vivo pathogenicity testing of yeasts. FEMS Immunol Med Microbiol. 2000;27(2):163–169.
39. Loh JM, Adenwalla N, Wiles S, et al. *Galleria mellonella* larvae as an infection model for group A *Streptococcus*. Virulence. 2013;4:419–428.
40. Kavanagh K, Reeves EP. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol Rev. 2004;28:101–112.
41. Shields RC, Zeng L, Culp DJ, et al. Genomewide identification of essential genes and fitness determinants of *Streptococcus mutans* UA159. mSphere. 2018;3(1):pii: e00031–18.
42. Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans.* Microbiology. 2008;154:3247–3255.
43. Freedman ML, Tanzer JM, Coykendail AL. The use of genetic variants in the study of dental caries. Animal models in cariology. In: Tanzer JM, editors.

Proceedings of the Symposium and Workshop on Animal Models in Cariology. Washington, DC: Information Retrieval Inc.; 1981. p. 247–269.

44. Ahn SJ, Qu MD, Roberts E, et al. Identification of the *Streptococcus mutans LytST* two-component regulon reveals its contribution to oxidative stress tolerance. BMC Microbiol. 2012;12:1–12.
45. Ahn SJ, Rice KC, Oleas J, et al. The *Streptococcus mutans* Cid and Lrg systems modulate virulence traits in response to multiple environmental signals. Microbiology. 2010;156:3136–3147.
46. Götz F. *Staphylococcus* and biofilms. Mol Microbiol. 2002;43:1367–1378.
47. Qiu W, Zheng X, Wei Y, et al. d-Alanine metabolism is essential for growth and biofilm formation of *Streptococcus mutans*. Mol Oral Microbiol. 2016;31 (5):435–444.
48. Spatafora G, Rohrer K, Barnard D, et al. A *Streptococcus mutans* mutant that synthesizes elevated levels of intracellular polysaccharide is hypercariogenic in vivo. Infect Immun. 1995;63(7):2556–2563.
49. Nilsson M, Rybtke M, Givskov M, et al. The *dlt* genes play a role in antimicrobial tolerance of *Streptococcus mutans* biofilms. Int J Antimicrob Agents. 2016;48:298–304.
50. Ahn SJ, Gu T, Koh J, et al. Remodeling of the *Streptococcus mutans* proteome in response to LrgAB and external stresses. Sci Rep. 2017;7(1):14063.
51. Inenaga C, Hokamura K, Nakano K, et al. A potential new risk factor for stroke: *Streptococcus mutans* with collagen-binding protein. World Neurosurg. 2018;113: e77–e81.
52. Misaki T, Naka S, Kuroda K, et al. Distribution of *Streptococcus mutans* strains with collagen-binding proteins in the oral cavity of IgA nephropathy patients. Clin Exp Nephrol. 2015;19(5):844–850.
53. Naka S, Hatakeyama R, Takashima Y, et al. Contributions of *Streptococcus mutans* Cnm and PA antigens to aggravation of non-alcoholic steatohepatitis in mice. Sci Rep. 2016;6:36886.
54. Tsai CJY, Loh JMS, Proft T. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence. 2016;7 (3):214–229.
55. Ahn SJ, Wen ZT, Burne RA. Effects of oxygen on virulence traits of *Streptococcus mutans.* J Bacteriol. 2007;189:8519–8527.

56. Guo L, McLean JS, Lux R, et al. The well-coordinated linkage between acidogenicity and aciduricity via insoluble glucans on the surface of *Streptococcus mutans*. Sci Rep. 2015;5:18015.
57. Beier D, Gross R. Regulation of bacterial virulence by two-component systems. Curr Opin Microbiol. 2006;9 (2):143–152.
58. Ahn SJ, Rice KC. Understanding the *Streptococcus mutans* Cid/Lrg system through CidB function. Appl Environ Microbiol. 2016;82(20):6189–6203.
59. Rice KC, Turner ME, Carney OV, Modification of the *Streptococcus mutans* transcriptome by LrgAB and environmental stressors. Microb Genom. 2017;3(2): e000104.
60. Mazda Y, Kawada-Matsuo M, Kanbara K, et al. Association of CiaRH with resistance of *Streptococcus mutans* to antimicrobial peptides in biofilms. Mol Oral Microbiol. 2012;27(2):124–135.
61. May JJ, Finking R, Wiegeshoff F, et al. Inhibition of the D-alanine: DAlanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. FEBS J. 2005;272 (12):2993–3003.
62. Richter SG, Elli D, Kim HK, et al. Small molecule inhibitor of lipoteichoic acid synthesis is an antibiotic for Gram-positive bacteria. Proc Natl Acad Sci U S A. 2013;110(9):3531–3536.
63. Paganelli FL, van de Kamer T, Brouwer EC, et al. Lipoteichoic acid synthesis inhibition in combination with antibiotics abrogates growth of multidrug-*resistant Enterococcus faecium.* Int J Antimicrob Agents. 2017;49 (3):355–363.

FIGURES

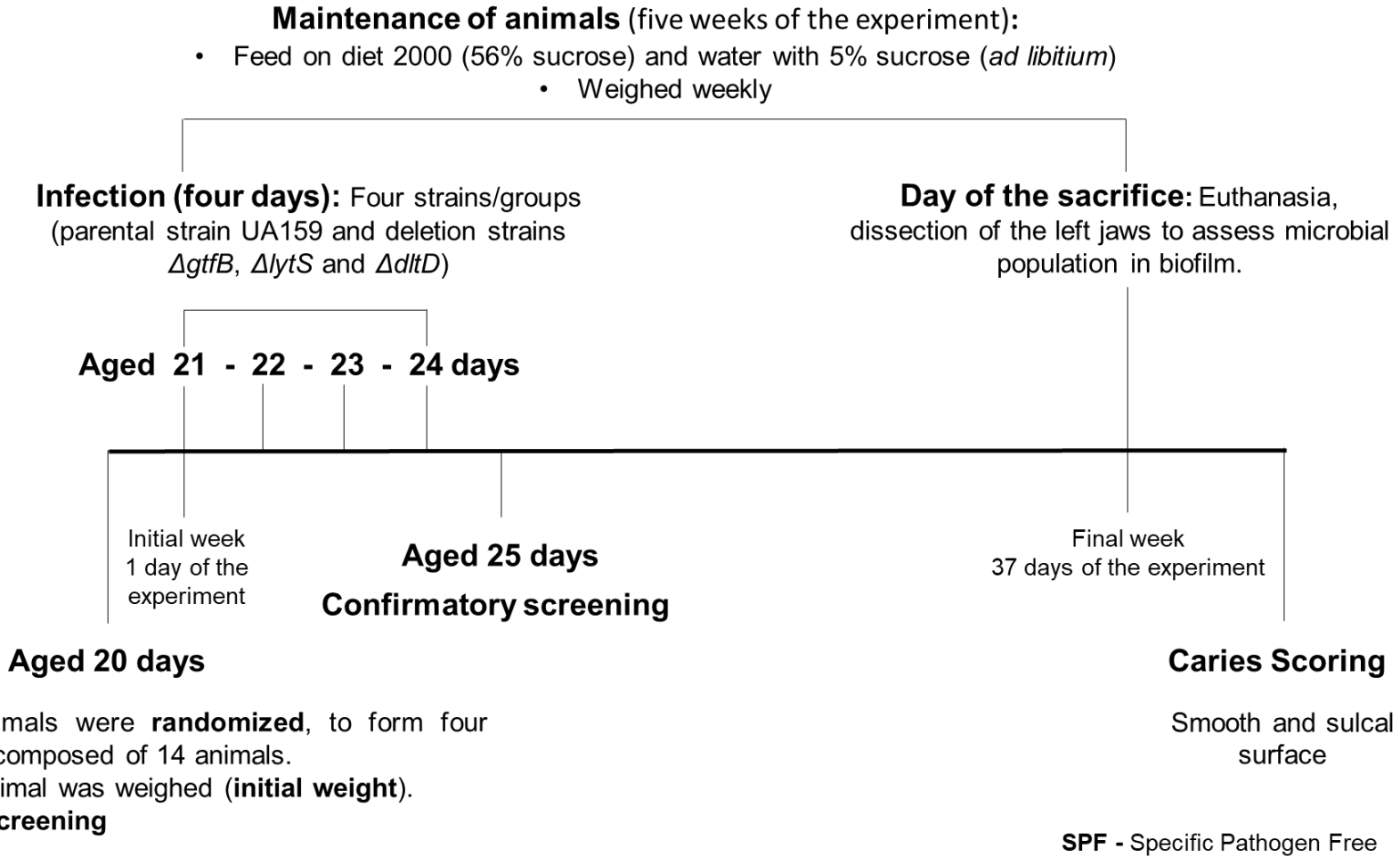

Figure 1. Experimental design for the rat model of dental caries.

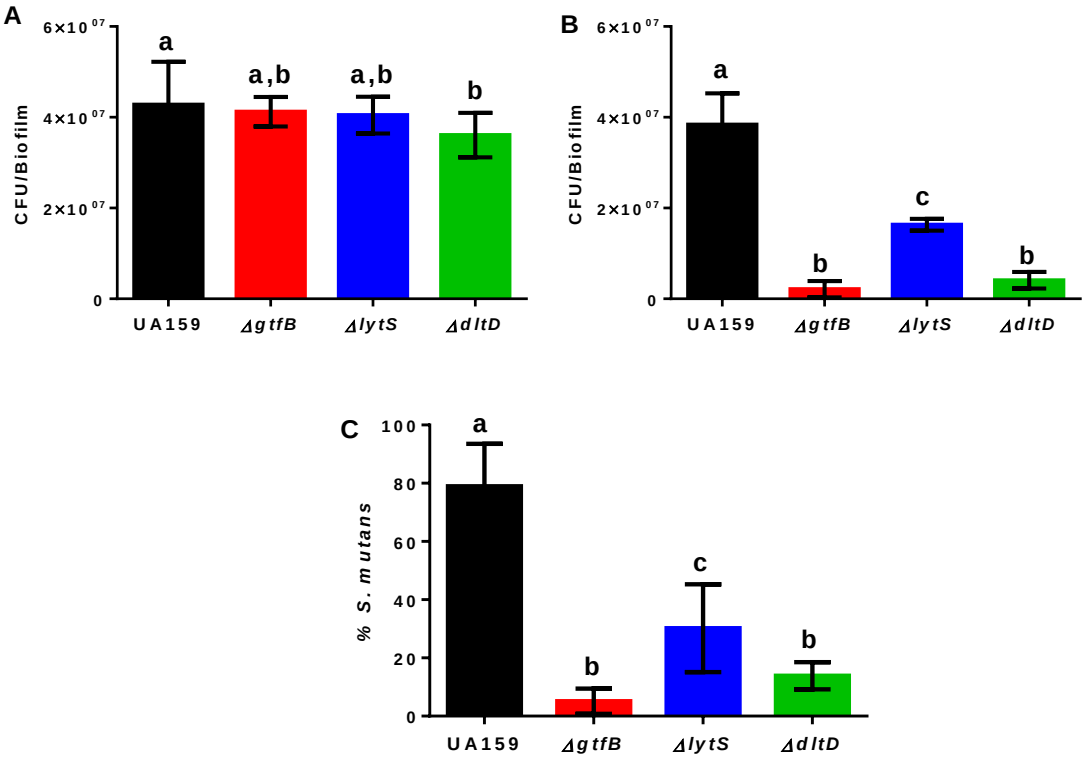

Figure 2. Microbial population recovered from the animals at the end of the caries study. The total cultivable microbiota (A) and *S. mutans* strains (B) derived from rats infected by parental strain UA159 and deletion strains Δ*gtfB*, Δ*lytS* and Δ*dltD*. (C) The percentage of *S. mutans* (%) in the total cultivable microbiota. Bars with the same letters indicate no statistical difference between the different strains in each graph ($p > 0.05$; one-way ANOVA, followed by Tukey's test). The data represented are the means, and the error bars correspond to the standard deviation (n = 14 per group).

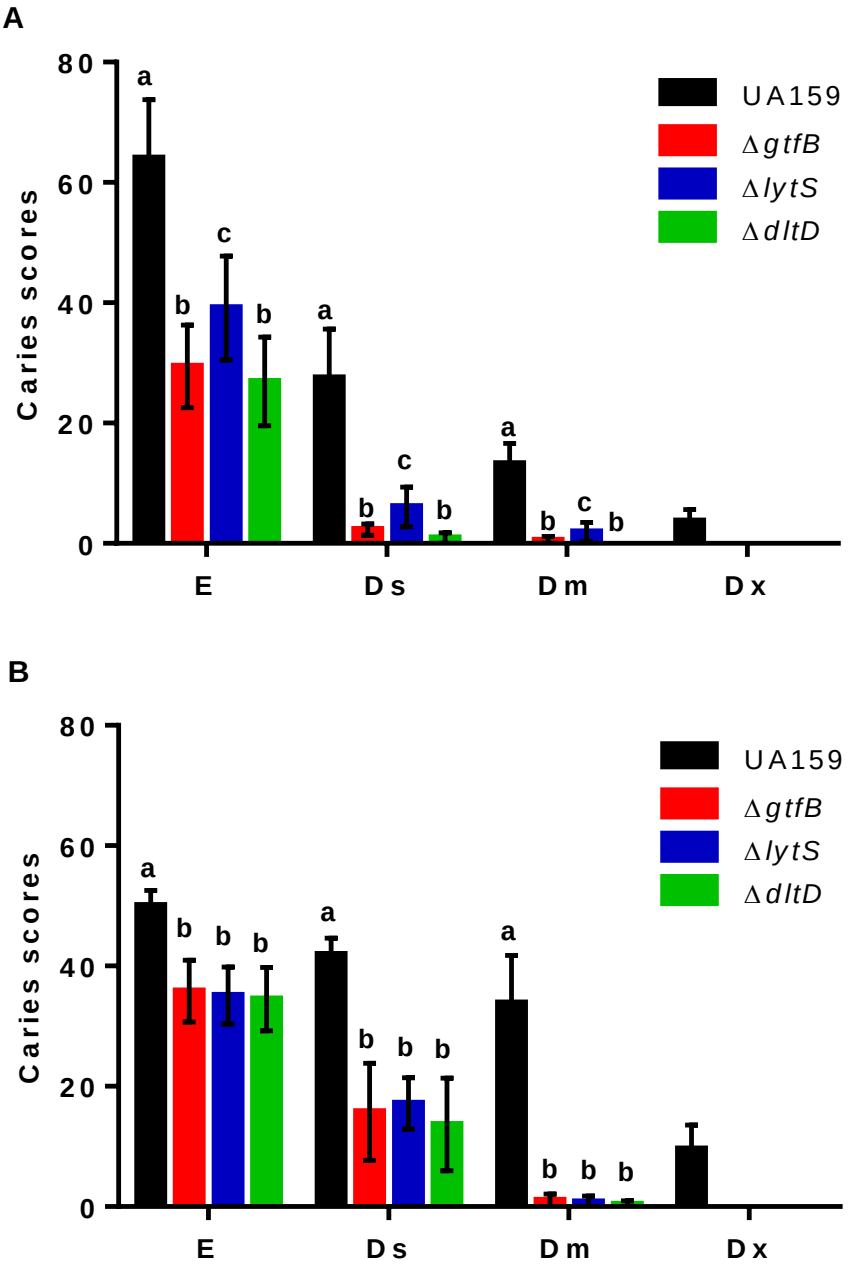

Figure 3. Influence of *S. mutans* strains on development and severity of dental caries on smooth (A) and sulcal (B) surfaces. The data displayed are the means, and the error bars correspond to the standard deviation (n = 14 per group). Bars with the same letters, by type of caries lesion, indicate no statistically significant differences between the strains ($p > 0.05$; two-way ANOVA, followed by the Tukey's test). In the Dx category for both smooth and sulcal caries, no bars, other than *S. mutans* UA159, are represented, since the three deletion strains *ΔgtfB*, *ΔlytS,* and *ΔdltD* did not yield this type of lesion. E: Enamel; Ds: light dentin caries; Dm: moderate dentin caries; and Dx: severe dentinal caries.

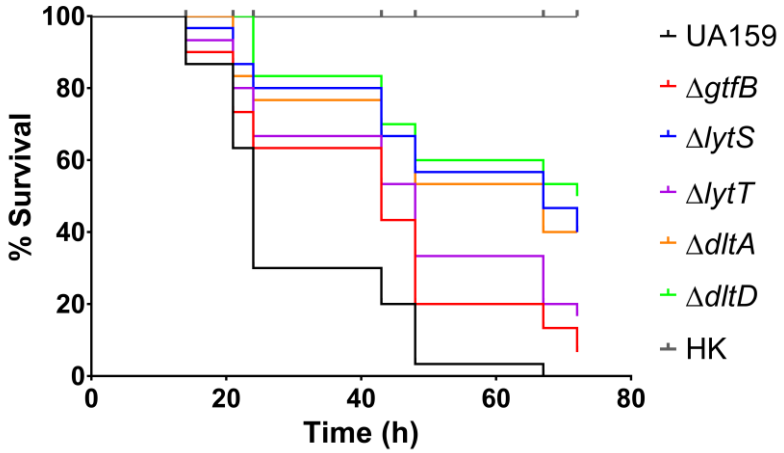

Figure 4. Survival of *G. mellonella* larvae after infection with *S. mutans* parental UA159 and deletion strains Δ*gtfB*, Δ*lytT*, Δ*lytS*, Δ*dltA* and Δ*dltD*. (B) Virulence of *S. mutans* strains displayed by Kaplan-Meier survival curves. Estimates of differences in survival were compared using the Matel-Cox Log-rank test. All larvae survived the heat-killed (HK) control of all strains tested, therefore, the data shown for HK are representative results from one strain.

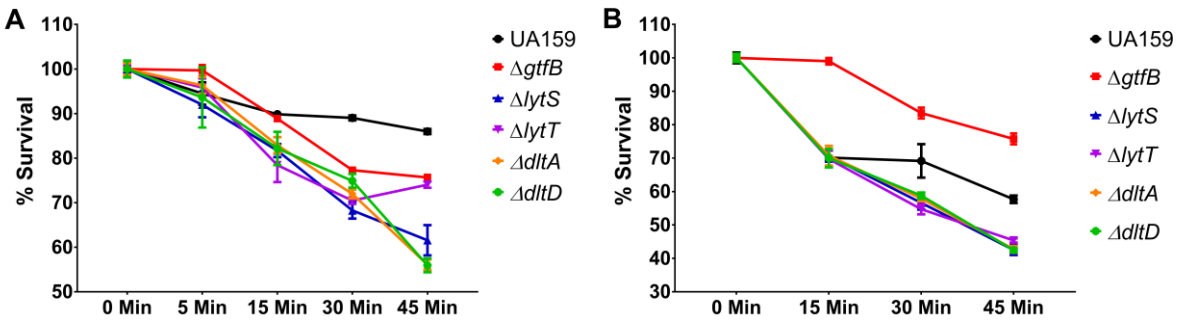

Figure 5. *S. mutans* parental UA159 and deletion strains Δ*gtfB*, Δ*lytT*, Δ*lytS*, Δ*dltA* and Δ*dltD* tolerance to hydrogen peroxide. The graphs show the different survival percentages of the strains population from planktonic culture (A) and biofilms (B) after longitudinal exposure to H_2O_2. The data shown are the means, and the error bars correspond to the standard deviation (n = 6 per group).

SUPPLEMENTAL MATERIAL

Table S1. Diet 2000.

Table S1. Contents of 2000 Diet (%)*
1% desiccated liver (DIFCO)
2% iodized salt (Cisne)
1% of protein in Alfalfa (20.29% protein-Accorsi Medicinal Plants)
5% of yeast powder extract (DIFCO)
7% of whole wheat flour (Renata)
28% of skimmed milk powder (La Serenissima)
56% of powdered icing sugar (Malvalério)

*The dietary material throughout the experiment came from the same manufacturing batch (Bowen *et al*., 1988).

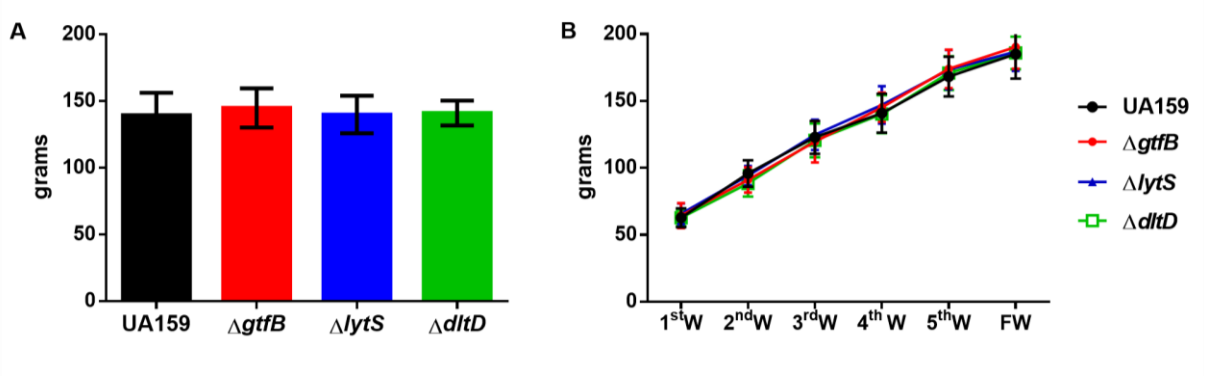

Figure S1. The weight of rats during the dental caries experiment. (A) The overall weight was similar between the different groups ($p > 0.05$, one-way ANOVA). The data represented are the means, and the error bars correspond to the standard deviation (n = 14 per group). (B) Longitudinal growth of rats. A significant increase in the weight of all animals occurred within each group over time ($p < 0.0001$ for each strain, two-way ANOVA and repeated measures, followed by Tukey's test). Thus, the Diet 2000 and other care during the maintenance period allowed all rats to grow equally well, as expected. These circumstances may provide an ideal environment for the implantation of microorganisms tested in the oral cavity.

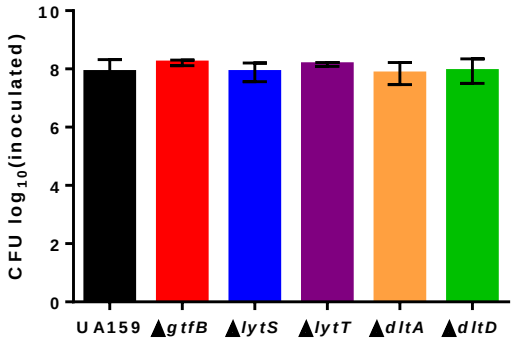

Figure S2. Quantity of *S. mutans* parental UA159 and deletion strains *ΔgtfB*, *ΔlytT*, *ΔlytS*, *ΔdltA* and *ΔdltD* inoculated into the hemocoel of *G. mellonella* larvae in the three experiments performed. The data represented are the means, and the error bars correspond to the standard deviation.

Table S2. Summary of *Galleria mellonella* larvae percentage survival over time.

Time (h)	Strains						
	UA159	*ΔgtfB*	*ΔlytS*	*ΔlytT*	*ΔdltA*	*ΔdltD*	HK
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
14	86.6	90.0	96.6	93.3	100.0	100.0	100.00
21	63.3	73.3	86.6	80.0	83.3	100.0	100.00
24	30.0	63.3	80.0	66.6	76.6	83.3	100.00
43	20.0	43.3	66.6	53.3	66.6	70.0	100.00
48	3.3	20.0	56.6	33.3	53.3	60.0	100.00
67	0.0	13.3	46.6	20.0	40.0	53.3	100.00
72		7.0	40.0	16.6	40.0	50.0	100.00

All larvae survived the heat-killed (HK) control of all strains tested, therefore, the data shown for HK are representative results from one strain.

3.2 Publicação 2*

Castillo Pedraza MC, Fratucelli ED, Ribeiro SM, Florez Salamanca EJ, Colin JS, Klein MI. **Modulation of lipoteichoic acids and exopolysaccharides prevents *Streptococcus mutans* biofilm accumulation.** J Oral Microbiol. Submission ID: 192990056; 08/02/19.

Modulation of lipoteichoic acids and exopolysaccharides prevents *Streptococcus mutans* biofilm accumulation

ABSTRACT

Background: Dental caries is a diet-biofilm-dependent disease. *Streptococcus mutans* contributes to cariogenic biofilms by producing an extracellular matrix rich in exopolysaccharides and acids. **Objective**: To determine the effect of topical treatments with compound 1771 (modulates lipoteichoic acid—LTA metabolism) and myricetin (affects the synthesis of exopolysaccharides) on *S. mutans* biofilms. **Design**: *In vitro S. mutans* UA159 biofilms were grown on saliva-coated hydroxyapatite discs, alternating 0.1% sucrose and 0.5% sucrose plus 1% starch. Twice-daily topical treatments were performed with both agents alone and combined with and without fluoride: compound 1771 (2.6 µg/mL), myricetin (500 µg/mL), 1771+myricetin, fluoride (250 ppm), 1771+fluoride, myricetin+fluoride, 1771+myricetin+fluoride, and vehicle. Biofilms were evaluated via microbiological, biochemical, imaging, and gene expression methods. **Results**: The compound 1771 alone yielded less viable counts, biomass, exopolysaccharides, and extracellular LTA. However, the combination 1771+myricetin+fluoride decreased 3 logs of bacterium counts, 60% biomass, >74% exopolysaccharides, and 20% LTA. The effect of treatments on extracellular DNA was not pronounced. The combination strategy affected the size of microcolonies and exopolysaccharides distribution and inhibited the expression of genes linked to insoluble exopolysaccharides synthesis. **Conclusions:** Compound 1771 prevented the accumulation of *S. mutans* biofilm; however, the effect was more pronounced when it was associated with fluoride and myricetin.

* Artigo escrito seguindo as normas bibliográficas da revista J Oral Microbiology (ANEXO A).

Keywords: Biofilm, *Streptococcus mutans*, compound 1771, myricetin, exopolysaccharides, lipoteichoic acids.

INTRODUCTION

Dental caries is a noncommunicable infectious disease that reaches a high percentage of the world population without distinction of age and is the main factor related to the loss of dentition [1-3]. Several factors interact for the disease occurrence [4-6], and its development is associated with the transition from a healthy biofilm to a pathogenic one [7,8]. Thus, strategies are needed to control cariogenic biofilm development.

Among several species that comprise the oral microbiota, *Streptococcus mutans* is considered an important etiological factor for dental caries in association with a sugar-rich diet [8,9]. The uniqueness of this species is its ability to orchestrate the cariogenic biofilm build-up by producing a three-dimensional (3D) extracellular matrix rich in exopolysaccharides (EPS) that impedes diffusion and creates microenvironments where metabolites such acids accumulate within the biofilm and at the interface biofilm/teeth surface [10], leading to the demineralization of teeth enamel [11]. This matrix also contains extracellular DNA (eDNA) and lipoteichoic acid (LTA) [12-14]. This bacterium also produces acids and withstands acidic niches [15].

Dietary sucrose and starch are substrate and acceptors for EPS synthesis by *S. mutans* exoenzymes glycosyltransferases (GtfB, GtfC, and GtfD) present on the pellicle and microbial surfaces enabling local accumulation of microbes on the teeth [16]. Also, fructans produced by a fructosyltransferase are a minor part of EPS in the matrix [16]. Over time, these EPS build a matrix that embeds the microbial cells forming a cohesive and diffusion-limiting milieu, creating acidic niches where cariogenic species thrive and cause acid-dissolution of teeth [10,11]. Moreover, EPS-rich extracellular matrix protects the microorganisms from antimicrobial therapies, and provides viscoelasticity to biofilms, hindering its mechanical removal [13].

eDNA cooperates with EPS in the early phases of biofilm development while LTA participates in the maturation of matrix and biofilm [14]. The deletion of *gtfB* gene affects the expression dynamics of eDNA-linked (*lytST, lrgAB, ccpA*) genes and markedly decrease the expression of *dltB* (from operon *dltABCD* linked to LTA

metabolism) and EPS-associated genes (*gtfBCD, gbpB, dexA*) [17]. Most of those genes have gene products involved with cell wall/membrane composition/structure, indicating that these cell components turnover may be affected, thereby influencing matrix and biofilm development. The inactivation of *lytST*, *dltAD*, and *gtfB* impaired *S. mutans* cariogenicity in a rodent model, and its virulence in a systemic infection model (*Galleria mellonella* larvae) [18]**.** Hence, strategies to modulate these genes and its products could affect *S. mutans* pathogenicity.

Classical caries prevention strategies include exposure to fluoride (aid in the remineralization process), restriction of dietary sugars (reduce substrate for microbial metabolism for EPS and acid production) and mechanical removal of dental plaque (brushing and flossing). In addition, chlorhexidine, a broad-spectrum bactericidal agent, suppresses mutans streptococci levels in saliva but is less effective against biofilms and has undesirable side effects [19]. Nevertheless, fluoride is the mainstay for caries prevention as it has led to the significant reduction in decay; however, it offers incomplete protection against caries and does not address the infectious aspects of the disease effectively, even though it can affect the glycolytic activity of streptococci [20]. Extensive efforts for fluoridated drinking water have been in place, but it does not reach the entire population, especially in Brazil [21]. Moreover, promoting fluoride toothpaste would retard caries in populations but would not eliminate it, as caries occurs in populations with good fluoride exposure [22]. Additionally, an increase in daily dosage may lead to fluorosis of teeth and bones [23,24].

The use of fluoride combined with bioactive agents can improve the individual action of fluoride and become an interesting strategy for cariogenic anti-biofilm therapies. In this context, myricetin is a flavonoid that inhibits *gtfBC* expression and EPS synthesis [25-27]. This flavonoid also affects *S. mutans* acidogenicity by hindering the F-ATPase activity and glycolytic pH-drop [25]. In addition, the compound 1771 is a small molecule (synthetic compound) that inhibits the synthesis of LTA in Gram-positive bacteria *Staphylococcus aureus* [28] and *Enterococcus faecium* [29]. Therefore, here it was investigated whether the combination of myricetin, compound 1771 and fluoride influences *S. mutans* biofilm development by interfering with the bacterium gene expression and metabolism that would hinder the extracellular matrix build-up.

MATERIAL AND METHODS

Experimental Design

An *in vitro* study with *Streptococcus mutans* was conducted to investigate the antimicrobial and antibiofilm activity of compound 1771 and myricetin. The antimicrobial activity was evaluated using planktonic cultures in microdilution assay to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Next, *S. mutans* single-species biofilm based on two batch culture approaches were used to investigate the anti-biofilm activity. First, the MIC and 2xMIC concentrations were used to evaluate the anti-biofilm effect of each agent using a polystyrene microplate biofilm model by determining *S. mutans* viable population (colony forming units or CFU/biofilm) and biomass (violet crystal method). After, 2xMIC of both agents alone and combined with and without fluoride were used for twice-daily topical treatments of biofilms formed on saliva-coated hydroxyapatite discs as substrate. The study was approved by the Institutional Ethical Committee (CAAE: 31717914.3.0000.5416) (ANEXO D).

In the second biofilm model, the eight experimental groups that corresponded to the distinct topical treatments were: compound 1771 (2.6 µg/mL), myricetin (500 µg/mL), compound 1771+myricetin, fluoride (250 ppm), compound 1771+fluoride, myricetin+fluoride, compound 1771+myricetin+fluoride, and vehicle (3.2% ethanol plus 0.02% dimethyl sulfoxide—DMSO). After 67 h, biofilms were evaluated to determine biofilm biomass, *S. mutans* viable counts, and extracellular matrix components (water-soluble and -insoluble EPS, eDNA, and LTA). Furthermore, after 46 h of biofilm development, gene expression of selected genes was performed, and confocal laser scanning microscopy was used to visualize and quantify the structural components (EPS and bacteria) of the biofilms. Moreover, during biofilm growth, the culture medium was changed twice daily, and the pH values of the spent medium were measured.

Three independent experiments were carried out in triplicate for the antimicrobial and anti-biofilm on polystyrene surface (n=9), while in duplicate for the anti-biofilm on saliva-coated hydroxyapatite surface (n=6). The data were statistically analyzed according to the factorial design of this study, considering each microplate well or hydroxyapatite disc as a statistical block. The discs were randomly assigned for each experimental group. Biochemical analyses of the extracellular matrix were performed

to verify how specific agents or combination of agents influenced biofilm development and consequent pathogenic potential. Our hypothesis was that interfering with EPS synthesis and cooperation with eDNA and LTA by using selected bioactive agents would hinder the development of *S. mutans* cariogenic biofilm. In addition, the cytotoxicity of each treatment and vehicle was investigated.

Test agents

Compound 1771 and myricetin were acquired via MolPort ordering service; compound 1771 was supplied by UkrOrg Synthesis Ltd (Cat. # PB25353228) while myricetin was supplied by AK Scientific Inc (Cat. # J10595). Sodium fluoride was purchased from Sigma-Aldrich Co. (St Louis, MO; Cat. # 71519). Stock solutions of each agent were prepared. Compound 1771 was dissolved in the combination of 84.5% ethanol and 15% DMSO at a final concentration of 2 mg/mL (4.68 mM). Myricetin was dissolved in 84.5% ethanol, with 1xPBS (Phosphate Buffered Saline, pH 7.2) at a final concentration of 15.912 mg/mL (50 mM).

For the antimicrobial activity assays (MIC and MBC), the concentrations used were 166.7-1.302 µg/mL for compound 1771 and 500-3.9 µg/mL for myricetin. For the antibiofilm assays on the polystyrene surface, the concentrations used were MIC and 2xMIC. However, the concentrations selected for twice-daily topical treatments of biofilms formed on saliva-coated hydroxyapatite discs as substrate were 2xMIC of both agents, alone and combined with and without fluoride: myricetin or Myr (500 µg/mL), compound 1771 or 1771 (2.6 µg/mL), compound 1771+myricetin or Myr+1771, myricetin+fluoride (250 ppm) or Myr+F, compound 1771+fluoride or 1771+F, compound 1771+myricetin+fluoride or Myr+1771+F, fluoride or F (250 ppm), and vehicle or V (3.2% ethanol plus 0.02% DMSO). Thus, the topical treatments, the test agents, including fluoride, were dissolved in 3.2% ethanol containing 0.02% DMSO just before carrying out the assays. The concentration of fluoride was selected based on the commercially available fluoride-based mouth rinse products [27,30,31].

Bacterial Strain and growth conditions

S. mutans UA159, serotype c (ATCC 700610) strain stocks stored at –80ºC in tryptic soy broth containing 20% glycerol were plated on blood agar plates (37°C, 5%

CO_2/95% air atmosphere or 5% CO_2, 48 h). Starter cultures were prepared by inoculating 5 to 10 colonies in tryptone-yeast extract broth (TY; 2.5% tryptone, 1.5% yeast extract, pH 7.0) containing 1% glucose tryptone broth and 1% glucose yeast extract, followed by incubation for 16 h (37°C, 5% CO_2). The starter cultures were diluted 1:20 in fresh TY+1% glucose and incubated until mid- exponential growth phase for the antimicrobial and antibiofilm assays.

Antimicrobial activity

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using broth microdilution following the Clinical and Laboratory Standards Institute [32], with some modifications. The *S. mutans* cultures at the mid-exponential growth phase were diluted in TY+1% glucose until reaching $2x10^6$ CFU/mL. Aliquots of 100 µL of these cultures were transferred to 96-well microplates. Next, treatments or vehicle were added, so the final bacterial load was $1x10^6$ CFU/mL, and the microplates were incubated (37°C, 5% CO_2, 24 h). In addition to treatments and vehicle, a microbial growth control without treatment was included; controls per treatment without microbial inoculation were also performed. Each treatment was performed in triplicates at three distinct experiments. After 24 h of incubation, visual inspection of the wells (turbidity: microbial growth, clear: no growth) and OD_{562nm} readings (ELISA plate reader, Biochrom Ez, Cambourne, UK) were performed to determine MIC. Also, an aliquot of each well was used for plating for a 10-fold serial dilution (10^{-1} to 10^{-5}) in saline solution (0.89% NaCl; Synth) to determine microbial cells viability by plating blood agar plates (37ºC, 5% CO_2, 48 h). After incubation, the colonies were counted to obtain CFU/mL and determine the MBC.

Antibiofilm assay using the polystyrene microplate model

The initial anti-biofilm assay was performed using the polystyrene microplate model to investigate whether compound 1771 presented antibiofilm activity. Even though it is known that myricetin has this activity [25-27], it was tested to ensure that the one from the acquired here also had this property. Therefore, *S. mutans* cultures at the mid-exponential growth phase were diluted in TY+1% sucrose until reaching

$2x10^6$ CFU/mL. Aliquots of 100 µL of these cultures were transferred to 96-well microplates. Next, treatments (MIC and 2xMIC) or vehicle were added, so the final bacterial load was $1x10^6$ CFU/mL, and the microplates were incubated (37°C, 5% CO_2, 24 h) [33]. In addition to treatments and vehicle, a microbial growth control without treatment was included; controls per treatment and medium without microbial inoculation were also performed. After 24 h of incubation, the microplates were placed on an orbital shaker at 75 rpm for 5 minutes (Quimis, São Paulo, BR). Next, the culture medium with unbound microbial cells was removed, and remaining biofilms were washed three times, with 0.89% NaCl solution to remove non-adhered cells.

The biomass of treated biofilms was assessed via violet crystal method. Briefly, the washed biofilms were stained with an aqueous solution of 0.1% violet crystal and incubated (25ºC, 35 min). Next, the wells were washed using Milli-Q water and air-dried for 60-90 min. Then, the violet crystal was eluted with 99% EtOH and incubation during 5 minutes on the orbital shaker (37ºC, 200 rpm). The eluted volumes were transferred to another microplate, and the OD_{570nm} was measured on an ELISA plate reader (Biochrom Ez). The readings per treatments and controls were converted into the percentage of biofilm biomass inhibition by each treatment and compared to vehicle control.

In addition, to verify the viable counts of the microbial population of treated biofilms, the washed biofilms were removed from each well using pipet tips and 0.89% NaCl solution and transferred to microtubes. Then, an aliquot of each biofilm suspension was used for a 10-fold serial dilution (10^{-1} to 10^{-5}) followed by plating on blood agar plates (37ºC, 5% CO_2, 48 h). After incubation, the colonies were counted to obtain CFU/mL and calculate the log of microbial growth inhibition by each agent, compared to vehicle control.

Biofilm formation on saliva-coated hydroxyapatite discs and topical treatments

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite (sHA) discs (surface area of 2.93 ± 0.2 cm^2, Clarkson Chromatography Products Inc., South Williamsport, PA, USA) in batch cultures for 46 and 67 h, as detailed elsewhere [14]. Saliva and pellicle preparation were performed as described before [34]. Saliva was donated by two volunteers who had not used antimicrobial treatments in the last three months. Each volunteer rinsed their mouth with 5 mL of Milli-Q water, then masticate

a piece of parafilm, collecting 5 mL saliva into a collection tube, which was then discarded. Next, the volunteers continued masticating the parafilm and collected saliva into an ice-chilled tube. The saliva samples from all volunteers were pooled and diluted 1:1 with adsorption buffer (AB buffer: 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM Phenylmethylsulfonyl Fluoride-PMSF, in dd-H_2O, pH 6.5). Saliva was centrifuged (3,220 x *g*, 20 min, 4°C; Centrifuge 5810R, Eppendorf) and the clarified portion was filtered sterilized (0.22 µm low protein binding polyethersulfone membrane filter). Saliva was used fresh for pellicle formation and medium preparation at the start of the experiment, and any remaining saliva was aliquotted and stored at -80°C until use for culture media preparation.

Saliva-coated HA discs were placed in a vertical position in a 24 well microtiter dish using a custom-made disc holder [14]. Next, these discs with salivary pellicle were dip-washed into wells containing AB buffer, and topically treated during 1.5 min with the seven tested treatments or vehicle control (eight experimental groups): (1) Myr; (2) 1771; (3) Myr+1771; (4) Myr+F; (5) 1771+F; (6) Myr+1771+F; (7) F; (8) V. The procedure for topical treatment consisted of dripping 250 µL of each treatment on the corresponding discs (two discs per treatment). The treated discs were dip-washed into wells containing AB buffer and transferred to wells containing *S. mutans* culture for biofilm formation (time 0 h). Here, the *S. mutans* cultures at the late-exponential growth phase were diluted in TY+0.1% sucrose and 25% saliva until reaching $2x10^6$ CFU/mL. The biofilms were incubated for 6 h (37°C, 5% CO_2), when the discs with initial biofilm were rinsed with 0.89% NaCl, treated with each corresponding treatment or control (as above), rinsed with 0.89% NaCl (to remove excess treatment), and transferred back to the corresponding culture media until biofilms were 19 h-old, when culture medium was changed.

The culture medium was changed daily at 8 am (TY+0.1% sucrose and 25% saliva) and 4 pm (0.5% sucrose + 1% starch and 25% saliva). After each media change, the pH of the spent media was measured. The biofilms were topically treated two hours after each culture change (Figure 1). The biofilms were grown until reaching two developmental phases: 46 h for gene expression assessment and 67 h for microbial population, biomass, biochemical characteristics of biofilm extracellular matrix, and confocal analyses.

Biofilm analyses

Determination of microbial population, biomass and biochemical characteristics of biofilm extracellular matrix

At 67 h of development, biofilms were processed for analyses following previously described protocols [34]. Briefly, biofilms were dip-washed into wells containing sterile 0.89% NaCl solution (saline solution). Each biofilm (disc) was transferred to a glass tube containing 1 mL of saline solution. Next, 1 mL of saline solution was used to wash the walls of each tube. The glass tubes with biofilms/discs were placed in a beaker and subjected to water-bath sonication for 10 minutes. A sterile metal spatula was used to scrape off any remaining biofilm from each disc surface, and the 2 mL of each biofilm suspension was transferred to a new 15 mL tube. Next, each glass tube was washed with 3 mL of saline solution, which were transferred to the tube containing the initial 2 mL, yielding 5 mL total biofilm suspension per biofilm/disc. Each biofilm suspension (5 mL) was sonicated using a probe at 7 w for 30 sec (Sonicador model Q125, QSonica).

An aliquot of each suspension (0.1 mL) was used for a 10-fold serial dilution to determine the number of CFU by plating on blood agar plates (37ºC, 5% CO_2, 48 h). The remaining volume (4.9 mL) was centrifuged (3,220 x *g,* 20 min, 4°C). The supernatant (with soluble extracellular matrix components) was transferred to a new tube, and the pellet (precipitate with the microbial cells and insoluble matrix components) was washed twice with 2.6 mL sterile Milli-Q water (3,220 x *g,* 20 min, 4°C). The supernatants generated during the two washes were combined with the first supernatant obtained, totaling 10 mL, which was used to isolate and quantify water-soluble EPS [35], eDNA [36], and LTA [14]. The pellet was suspended in 2.55 mL of Milli-Q water, of which 0.5 mL was used for quantification of insoluble dry-weight (biomass) and 1 mL for the isolation and quantitation of water-insoluble EPS (or alkali-soluble polysaccharides) [35].

Laser scanning confocal fluorescence microscopy imaging and computational analyses of treated biofilms

Biofilms were formed and treated as described above. However, 1 μM Alexa Fluor™ 647-labeled dextran conjugate (absorbance/fluorescence emission maxima of 647/668 nm; Molecular Probes, Carlsbad, CA, USA) was added to the culture medium

at the beginning of, and during the development of the biofilms [37]. This strategy enables the incorporation of labeled dextrans into EPS during its synthesis process and matrix build-up. When the biofilms reached 67 h of development, the discs were dip-washed into wells containing with 0.89% NaCl and transferred to wells containing 0.89% NaCl solution and SYTO™ 9 (485/498 nm; Molecular Probes), which is a green fluorescent nucleic acid marker for visualization of bacteria [37]. The imaging of the three-dimensional structure of these biofilms was performed using a Zeiss LSM 780 microscope (Zeiss, Jena, Germany), fitted with a 20X objective lens. Each biofilm was scanned at three randomly selected positions, and a series of confocal images were generated by optical sectioning at each of these positions. The images were analyzed using Amira 6.0.1 software (Mercury Computer Systems Inc., Chelmsford, MS, USA) for 3D reconstruction of EPS and bacteria [10]. Furthermore, each image was analyzed using COMSTAT version 2 software [38] for quantification of total bacteria content and EPS matrix (bio-volume), and percent of coverage per area from the interface substrate/biofilm (hydroxyapatite disc) to the top (outer layer) of each biofilm.

Gene expression analysis of treated S. mutans biofilms

Five treatments for biofilms were chosen to evaluate *S. mutans* gene expression based on the data from biochemical analyses and 3D structure of biofilms: Myr, 1771, Myr+1771+F, F, and V (control). The *S. mutans* assessed genes were associated with EPS (*gtfB* and *gtfC*), eDNA (*lrgA*), LTA (*dltB*, *dltD*, and *SMU.775*), and tolerance to acid (*atpD)* and oxidative (*nox1*) stresses [10,17,39,40]. Biofilms were grown and topically treated as described earlier. At 46 h of growth that corresponds to 1 h after treatments (at 45 h), biofilms were removed and processed for total RNA isolation, followed by cDNA synthesis and quantitative PCR (qPCR) following the MIQE guidelines [41].

The biofilms were dip-washed into wells containing sterile 0.89% NaCl solution (saline solution). Each biofilm (disc) was transferred to a glass tube containing 1 mL of RNAlater™ Stabilization Solution (Ambion, Austin, TX, USA). Next, 1 mL of RNAlater™ was used to wash the walls of each tube. The glass tubes with biofilms/discs were placed in a beaker and subjected to water-bath sonication for 10 minutes for biofilm detachment from discs. A sterile RNAse-free metal spatula was used to scrape off any remaining biofilm from each disc surface. Each biofilm

suspension was transferred to a new centrifuge tube, and two mL of RNAlater™ were used to rinse the glass and the discs; this volume was recovered and stored in the corresponding centrifuge tubes. These biofilm suspensions were stored at −80°C until RNA isolation.

RNA was extracted as described elsewhere [42]. Briefly, biofilm suspension in RNAlater™ were diluted 1:1 with 1xPBS (pH 7.2) and centrifuged (3,220 x *g*, 20 min, 4°C). The supernatant was discarded, and the resulting pellet was resuspended in 5 mL of 1xPBS, followed by sonication with a probe for 30 sec at 7 w (Sonicador model Q125); this procedure was repeated twice. After, the pellet was resuspended in 750 µL NAES (50 mM sodium acetate buffer, 10 mM EDTA and 1% SDS; pH 5.0; Ambion) and 750 µL acid phenol (Ambion). This mixture was transferred to tubes containing glass beads, followed by mechanical disruption using a Bead-beater for 40 seconds (Biospec Products) and cooling samples by placing them on ice for 1 min. This homogenization procedure was repeated twice, and the samples were centrifuged (15,480 x *g*, 5 min, 4°C; Centrifuge 5430R, Eppendorf). Then, RNA was extracted using the phenol-chloroform acid separation method and purified with DNAse in column (RNeasy Micro Kit, Qiagen, Austin, TX, USA) and solution (Turbo DNase; Ambion, Austin, TX, USA). DNAse was removed using the RNeasy MinElute clean-up kit (Qiagen). After purification, the total RNA amount (OD 260nm) and purity (OD 260/280 ratio) were verified (DS-11 + Nano-spectrophotometer). The integrity of the purified RNA was determined by 1% agarose gel electrophoresis. RNA was diluted to a concentration of 100 µg/µL and stored at -80°C until cDNA synthesis.

cDNA synthesis was performed in duplicate per sample using 0.5 µg of total RNA and the iScript kit (Bio-Rad Laboratories, Inc., CA, USA). Negative controls were made without using reverse transcriptase to determine whether there was DNA contamination. Reactions were incubated using CFX96 Touch ™ Real-Time PCR Detection System (Bio-Rad), with the cycle: 25°C / 5 min, 42°C / 30 min, 85°C / 5 min, 4°C ∞. The cDNA samples were stored at -20°C until used for the quantification of gene expression (qPCR).

qPCR analyses were performed using specific primers from the literature [10,17,39,40]. cDNA was diluted 1:5 for specific genes and 1:1,000 for 16S rRNA gene (used for normalization of specific gene expression); cDNA negative controls were not diluted. cDNA and negative controls were amplified by a CFX96 System (Bio-Rad)

using specific primers and iQ SYBR Green supermix (Bio-Rad). A standard curve was plotted for each primer set as detailed elsewhere [43]. The reactions were run using the following cycle in a CFX96 System (Bio-Rad) equipment: step 1 (1X) 95 °C / 3 min; Step 2 (35X) 95ºC / 15 sec, 58ºC / 30 sec, 68ºC / 15 sec (data collection); Step 3 (1X) 95 °C / 1 min; Step 4 (1X) 55 °C / 1 min; Step 5 (80X) 55ºC / 1 min, for analysis of the melting curve. The standard curves were used to transform the Quantification Cycle (Cq) values to relative numbers of cDNA molecules. Relative expression was calculated by normalizing each gene of interest to the 16S rRNA reference gene. Next, these values were compared to those from biofilms treated with vehicle-control to determine the fold-change in gene expression.

Cytotoxicity of treatments

Oral keratinocytes NOK-si lineage [44] according Standard International Organization (ISO) 10993-5 [45], were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY, USA) with 2 mM glutamine; containing 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY), penicillin G (10.000 µg/mL), streptomycin (10.000 µg/mL) and amphotericin (25 µg/mL) (Invitrogen). The culture was incubated (37°C, 5% CO_2). The cells were grown to confluency (90%), washed with 1X phosphate buffer (140 mM NaCl, 3.0 mM KCl, 4.30 mM Na_2HPO_4, 1.40 mM KH_2PO_4, pH 7.0), removed with trypsin (0.05%) / EDTA solution (0.53 mM) (Invitrogen), and then centrifuged (400 x *g*, 5 min). The cells were resuspended in the same culture medium and replated. The medium was changed every two or three days. For the experiments, cells between the 3rd and 8th passage were used. Cells were counted in Neubauer's chamber and plated in 96-well microplate wells ($2x10^4$ cells well). The plates were incubated for 24 h (37°C, 5% CO_2).

Next, the cytotoxicity resulting from the presence of treatments (agents and vehicle control), death control (0.11% Triton X-100), and untreated control (cell viability control) on monolayer cells was determined by the colorimetric assay of viability cell MTT [3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (Sigma). This assay was performed using cell culture in monolayer, 1 h after contact with treatments and controls included in the culture medium. After the incubation period, the cells were washed with 500 µL of 1xPBS (pH 7.4) and incubated (37°C, 5% CO_2, 4 h) with 250 µL of MTT solution (5 mg/mL). Then the forming crystals were solubilized in 250 µL of

2-propanol added to each well. Spectrophotometric measurements were performed at a wavelength of 562 nm. Two experimental occasions were performed with 4 replicates per occasion (n=8). The data obtained were converted into a percentage of viable cells and compared to the control without treatment (control of cell viability).

Statistical analyses

The data were analyzed to evaluate whether the tested treatments affected *S. mutans* biofilm using Prism 7 software (GraphPad Software, Inc. 2018). The analyses were performed using descriptive and inferential statistic according to the distribution (Shapiro-Wilk test of normality; $\alpha = 0.05$). The antimicrobial activity data of myricetin and compound 1771 showed a normal distribution and were evaluated with one-way ANOVA, Dunnett's post-test ($\alpha = 0.05$). The anti-biofilm activity data of myricetin and compound 1771 did not present normal distribution, so it was evaluated with the Kruskal-Wallis test, with Dunn's post-test ($\alpha = 0.05$). The data obtained for topically treated biofilms presented a normal distribution; thus, data for the viable counts, biomass, matrix components (water-soluble and -insoluble EPS, eDNA, and LTA), biovolume quantification of bacteria and EPS were evaluated by one-way ANOVA, followed by test Tukey's multiple comparisons ($\alpha = 0.05$). The gene expression data were normalized by the 16S rRNA gene data, followed by an analysis of the fold change relative to the vehicle. Data for gene *gtfB*, *gtfC*, *IrgA,* and *SMU.775* did not present normal distribution and were examined using the Kruskal-Wallis test, followed by Dunn's post-test ($\alpha = 0.05$). Meanwhile, data for genes *atpD*, *nox1*, *dltB,* and *dltD* presented normal distribution and were evaluated via one-way ANOVA, followed by test Tukey's multiple comparisons ($\alpha = 0.05$). The data from cytotoxic assays presented normal distribution and were evaluated using one-way ANOVA, followed by Tukey's multiple comparisons test ($\alpha = 0.05$).

RESULTS

Antimicrobial activity

The MIC and MBC values were 1.302 µg/mL for compound 1771; while for myricetin, these values were 250 e 500 µg/mL, respectively. Figure 2 depicts the *S. mutans* viable population recovered at distinct concentrations of agents.

Antibiofilm activity using the polystyrene microplate model

The anti-biofilm effect of MIC and 2xMIC for compound 1771 and myricetin are shown in Figure 3. Both agents reduced the counts of *S. mutans* viable population by > 4 logs and biomass by >99% ($p<0.0001$; Kruskal-Wallis test with Dunn post-test).

Effect of topical treatments on biofilms grown on saliva-coated hydroxyapatite discs

pH of the spent culture media

The pH of spent media was measured after the culture medium changes (19, 27, 43 and 51 h) and when the biofilms were removed for processing and analyses (67 h) (Figure 4). At 19 h the biofilms treated with 1771 had the highest pH value (it was less acidic) when compared to the treatments Myr+F ($p=0.0009$), 1771+F ($p=0.0017$), Myr+1771+F ($p=0.0027$), and V ($p=0.0466$) (one-way ANOVA, followed by Tukey's test). At 27 h, there were no differences between treatments and vehicle. However, at 43, 51 and 67 h, vehicle group presented the lowest pH value *versus* all treatments ($p<0.0001$; one-way ANOVA, followed by Tukey's test). The combination strategy Myr+1771+F yielded the highest pH value compared to Myr+1771 and F at 43 h, while it was less acid *versus* all other treatments at 67 h ($p\leq0.0356$; one-way ANOVA, followed by Tukey's test). Additionally, at 67 h F treatment yielded the highest pH value when compared to Myr, 1771, Myr+1771, and Myr+ F ($p<0.0001$; one-way ANOVA, followed by Tukey's test). Furthermore, the agents combined with fluoride lead to higher pH values in the spent media (*vs*. vehicle or agents alone or combined). However, the pH values were similar for all treatments at 46 h, when biofilms were processed for gene expression analysis (these data are not depicted in the graph because they are from five experimental groups selected for this essay).

Viable counts of S. mutans and biomass

The quantification of viable *S. mutans* numbers is shown in Figure 5A. All treatment with agents alone or combined with or without fluoride reduced the bacterial population in comparison with the vehicle ($p<0.0001$; one-way ANOVA, followed by Tukey's test). The compound 1771 alone reduced 1 log compared to the vehicle. However, this effect was most pronounced in the biofilms treated with the combination strategy Myr+1771+F, which had 3 logs difference from the vehicle ($p<0.0001$; one-

way ANOVA, followed by Tukey's test). Furthermore, a similar trend was observed for the biomasses of the insoluble portion of *S. mutans* biofilms (Figure 5B); all treatments were able to decrease the values *versus* the vehicle-treated ($p<0.0001$; one-way ANOVA, followed by Tukey's test). However, the highest reduction in biomass was observed for biofilms treated with the combination of Myr+1771+F when compared to all other treatments and vehicle ($p<0.0001$; one-way ANOVA, followed by Tukey's test), showing a ≅60% reduction *versus* the vehicle.

Extracellular matrix components

The quantification of four components of the extracellular matrix is depicted in Figure 6. Regarding EPS, all treatments yielded biofilms with lower quantities of both water-soluble and -insoluble EPS compared to vehicle-treated biofilms (Figure 6A and 6B; $p<0.0001$; one-way ANOVA, followed by Tukey's test). However, the combination of Myr+1771+F was more effective in hindering the accumulation of water-insoluble EPS (a marker of cariogenic biofilms; ≅87.87±4.23%) and water-soluble EPS (*vs*. all other treatments; ≅73.88±10.83%), although the effect of Myr alone was also pronounced for both types of EPS. In contrast, the effect of treatments on eDNA and LTA amounts was not as noticeable than EPS (Figure 6C and 6D). However, the four treatments containing compound 1771 yielded about 15 to 20% less LTA in the matrix than the other treatments, especially fluoride ($p\leq0.0303$; one-way ANOVA, followed by Tukey's test).

3D structure, biovolume and percent coverage distribution of bacteria and EPS

Representative images of the 3D structure of biofilms are shown in Figure 7. Biofilms treated with vehicle presented agglomerations of well-defined large microcolonies protected by the EPS in the extracellular matrix. In contrast, the biofilms treated with the combination Myr+1771+F presented smaller microcolonies that were spread out across the disc surface, with a less defined EPS matrix. The treatments Myr+1771 and F also yielded fewer microcolonies, but they appear to be closer together, and eventually, larger microcolonies are observed. The treatments Myr, 1771, Myr+F, and 1771+F presented a similar architecture, with large microcolonies and small microcolonies (interspaced between the larger ones) spread out on the

surface of the disc. Thus, in general, the 3D structures of the treated biofilms were different, but the most striking differences were observed for biofilms treated with the combination of Myr+1771+F *versus* the vehicle-treaded. This observation can be reinforced by the amount of biovolume (biomass) of bacteria and EPS (Figure 8) and the distribution profile of bacteria and EPS (Figure 9). The percentage of coverage per area from the interface substrate/biofilm (hydroxyapatite disc) to the top (outer layer) of each biofilm demonstrated that the vehicle exhibited a higher percentage of coverage compared to the other treatments, especially the combination of Myr+1771+F.

S. mutans gene expression

The effect of five selected treatments on *S. mutans* gene expression is depicted in figure 10. The *gtfB* and *gtfC* genes that encode exoenzymes that synthesizes exopolysaccharides presented lower expression in biofilms treated by the combination of Myr+1771+F (*vs*. V; $p \leq 0.0005$; Kruskal-Wallis test, with Dunn's post-test). However, the expression of *gtfB* was also repressed by fluoride ($p \leq 0.0092$), while the expression of *gtfC* was down-regulated by Myr and 1771 alone. In contrast, *lrgA* and *SMU.775* genes were induced by the combination of Myr+1771+F and F alone (*vs*. V; $p \leq 0.0005$; Kruskal-Wallis test, with Dunn's post-test). Nonetheless, the expression of *lrgA* and *SMU.775* were similar for biofilms treated with compound 1771 alone, myricetin alone, and vehicle-control ($p > 0.05$). In addition, there were no significant differences in the expression of genes *nox1*, *dltB,* and *dltD* genes for the tested treatments and vehicle. However, the fluoride alone significantly decreased the expression of *atpD*, involved in *S. mutans* acid tolerance.

Cytotoxicity of treatments

The exposure of NOK-si lineage cells for 1 h to the treatments with Myr+1771 and Myr+F decreased cell viability in 13 and 25%, respectively, compared to the viability control (Figure 11). Nevertheless, the model used here had cells organized as a monolayer and not a tissue with a three-dimensional organization seen in the oral mucosa. Also, a formulation used in the oral cavity will have a shorter exposure time, as tested here for topical treatments (1.5 min).

DISCUSSION

Approaches combining bioactive agents with fluoride for preventing cariogenic biofilm development have been proposed, including compounds that affect the production of EPS-rich matrix [27,46]. Taking in consideration that EPS interacts with eDNA and LTA the extracellular matrix [14] and that meddling with genes associated with EPS, eDNA and LTA reduces the cariogenicity of *S. mutans* [18], the current study investigated whether a compound that targets LTA metabolism in Gram-positive bacteria and works as an antimicrobial [28,29] would have an effect when used for topical treatment and whether combining this agent with a flavonoid that hinders EPS production and fluoride would have a more pronounced effect in hampering *S. mutans* biofilm development.

The topical application of compound 1771 and myricetin alone prevented the accumulation of *S. mutans* biofilm *in vitro*. However, the effect was more pronounced when compound 1771 was associated with myricetin and fluoride, affecting the amount of matrix components and the 3D architecture of biofilms. The combination of compound 1771+myricetin+fluoride reduced 3 logs of *S. mutans* counts, ≅60% of the biomass, ≥74% water-soluble and -insoluble EPS, and ≅ 20% of LTA in the matrix (*vs*. vehicle). Nevertheless, the slight changes in the quantities of eDNA and LTA could affect the 3D organization of the biofilms, as both biomolecules interact with EPS in the matrix at distinct stages of biofilm development; eDNA at the early phases while LTA during the later phases of biofilm development [14]. Moreover, the agents combined with fluoride lead to higher pH values in the spent media (*vs*. vehicle or agents alone or combined). Inhibition of the dynamic processes that occur within a biofilm can minimize or avoid the damage caused by it on teeth surfaces and prevent dental caries.

EPS synthesis and its 3D organization in the matrix is a determinant factor in the etiology of dental caries [11]. In the presence of dietary carbohydrates, EPS are mainly glucans produced by *S. mutans* glucosyltransferases GtfB, GtfC, and GtfD, encoded by *gtfB*, *gtfC,* and *gtfD,* respectively [16]. Here, the expression of *gtfB* and *gtfC* genes was lower in biofilms treated by the combination of Myr+1771+F. However, the expression of *gtfB* was also repressed by fluoride, and the expression of *gtfC* was down-regulated by Myr and 1771 alone. These differences may be because GtfB produces water-insoluble EPS, while GtfC produces both water-soluble and -insoluble

EPS, and the location where these exoenzymes bind preferentially. GtfB has more affinity to the bacterial cell wall and GtfC to the salivary pellicle.

Moreover, repression of *gtfB* and *gtfC* may explain the reduction of water-soluble and -insoluble EPS in the matrix. As GtfB binds to the surface of the microorganisms and the surface of hydroxyapatite [47]; the EPS formed on the microbial surface improves the interactions between the microorganisms, cellular aggregation, and increases the cohesion of the biofilm [48]. Interestingly, the expression of the genes associated with the synthesis, binding, and remodeling of exopolysaccharides is reduced in a deletion strain of the *gtfB* gene [17]. Therefore, the adverse effect on *gtfB* gene expression justifies the low content of water-insoluble EPS and biomass in biofilms treated with myricetin, compound 1771 combined or not with fluoride; resulting in a biofilm with disadvantages in the survival and persistence of virulent organisms. Fluoride makes an active contribution to the repression of *gtfB*, regardless of concentration (125 or 250 ppm F) [26].

Although eDNA quantification showed statistically significant differences for the combination of myricetin, 1771 compound with or without fluoride, compared to the effect of treatments on EPS, myricetin and compound 1771 affected the amount of eDNA to a lesser extent. This matrix component is important in the construction, 3D architecture, and stability of the matrix [13,14] and in the virulence of *S. mutans* biofilms [18]. Surprisingly, the expression of *lrgA* was higher in biofilms treated with the combination of Myr+1771+F and F alone; thus, this behavior may be associated with fluoride. *lrgA* gene is involved in *S. mutans* cell-wall remodeling and autolysis processes that can yield eDNA [49,50]. Therefore, an increase in expression of *lrgA* may indicate that these treatments trigger cell wall remodeling and/or to autolysis. Also, eDNA can be actively secreted from live *S. mutans* cells via additional metabolic pathways involved in protein secretion and components for protein insertion into membranes (*i.e.*, Ffh, YidC1, and YidC2) [12]. However, eDNA may be released but may not be incorporated in the matrix if treatments are impeding EPS synthesis and/or binding; consequently, eDNA as a soluble component could be in the spent medium [51,52], or being consumed as a nutrient during the periods with low carbohydrate concentration in the medium and not be part of the structure of biofilms thereby affecting the 3D architecture of treated biofilms (*vs*. vehicle control).

In addition, the LTA content in the matrix of the biofilms treated was not that different, but LTA quantities were significantly reduced in those subjected to the four treatments containing compound 1771 (1771 alone, Myr+1771, 1771+F, and Myr+1771+F). A small reduction in LTA may also be contributing to alteration in the biofilm architecture, *S. mutans* viable population, biomass, and EPS in the matrix. Moreover, the expression of gene *SMU.775* was induced by the combination of Myr+1771+F and F alone while 1771 or myricetin alone yield similar expression to the vehicle-control. Thus, the induction of SMU.775 could be because of fluoride per se, as for lrgA. *SMU.775* is a hypothetical gene with homology to the LTA synthase from *S. aureus* and so, could be involved in the metabolism of LTA [13]. However, there were no significant differences in the expression of genes *dltB* and *dltD* for the tested treatments and vehicle. Hence, the tested agents alone or combined with fluoride did not affect the expression of operon *dltABCD* genes involved in the addition of D-alanine residues during LTA synthesis [53]. Several studies demonstrated that interfering with the D-alanization process of LTA affect the cells surface charge and cells susceptibility to antimicrobials [54-56]. Nevertheless, it is possible that the compound 1771 interfered with the LTA metabolism and affected the cell surface charge and/or composition, thereby, interfering with how EPS would bind to cells and mediate the formation of microcolonies and EPS distribution in the 3D structure of treated biofilms.

During biofilm development, the microbial cells produce components for the extracellular matrix, and these components attach to the cell wall and to the substrate (i.e., the surface where biofilm grows on) [57]. Thus, of alterations in the cell wall turnover and composition (and charge), or quality and quantity of matrix' components can interfere with cell-cell binding, cell-matrix, cell-matrix-dental surface, modifying the 3D architecture of biofilms and its cariogenic potential. Furthermore, the microbial cells in biofilms with distinct 3D structure may respond differently to environmental stresses. *S. mutans* possess a vast arsenal to cope with these stressors, especially acidic environment, and oxidative stresses.

Here, the expression of nox1 related to the oxidative stress response was similar between the tested treatments and vehicle control. However, the F slightly decreased the expression of *atpD*, involved in *S. mutans* acid tolerance. It has been shown that fluoride affects the glycolytic activity in cells and inhibits intracellular enzymes when it interferes with membrane proton permeability [58], which would affect the tolerance of

S. mutans to the acidic microenvironments within a biofilm [24,26]. Myricetin also contributes aciduricity via inhibition of glycolytic activity by altering membrane permeability [26]. However, here, when myricetin and 1771 were used alone or in combination with fluoride to treat biofilms, these agents did not interfere with the expression of *atpD*, which could be because of the concentrations used, exposure time and/or their effect would be detected at a different biofilm developmental phase. Nevertheless, it was demonstrated that insoluble glucans are an essential factor linking acidogenicity with aciduricity [59]; thereby the combination tested here would not only influence the matrix composition, viable counts but how the cells in the biofilm respond to an acidic environment during biofilm growth.

The combination strategy yielded lower EPS matrix production and the amount of small microcolonies. The microcolonies morphology and the EPS distribution influence the diffusion of the metabolites in biofilms and the action of antimicrobial agents on the microorganisms. It was demonstrated that within larger microcolonies, acidic niches are created, with pH values 4.5 - 5.5, increasing adhesion, strengthening the biofilm structure, and decreasing cell dead by cationic chlorhexidine [10]. The persistence of these acidic microenvironment leads to dental demineralization. Thus, the use of myricetin and compound 1771 combined with fluoride could be an ally in reducing the pathogenicity of the dental biofilm. Usage of myricetin and compound 1771 is not intended to eliminate fluoride but to improve the cariostatic efficacy of this product due to advantages of fluoride. Although the *S. mutans* biofilm model employed here for twice-daily topical treatments does not mimic the biological interaction of microorganisms within a cariogenic dental plaque, it shows that the tested agents interfered with critical cariogenic properties. Future studies should dissect how the proposed combination of Myr+1771+F affects the complex oral microbiota and prevents carious lesions *in vivo*, and whether increasing the concentration of agents and/or exposure/retention time (e.g., via drug-delivery systems).

In summary, the topical application of a compound that modulates lipoteichoic acid metabolism prevented the accumulation of *S. mutans* biofilm *in vitro*. Moreover, the effect was more pronounced when compound 1771 was associated with fluoride and a flavonoid.

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REFERENCES

1. Marcenes W, Kassebaum NJ, Bernabé E, Flaxman A, Naghavi M, Lopez A, et al. Global burden of oral conditions in 1990-2010: a systematic analysis. J Dent Res. 2013; 92(7): 592–7.
2. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of untreated caries: a systematic review and metaregression. J Dent Res. 2015; 94(5): 650-8.
3. Kassebaum NJ, Smith AGC, Bernabé E, Fleming TD, Reynolds AE, Vos T, et al. Global, Regional, and National Prevalence, Incidence, and Disability-Adjusted Life Years for Oral Conditions for 195 Countries, 1990-2015: A Systematic Analysis for the Global Burden of Diseases, Injuries, and Risk Factors. J Dent Res. 2017; 96(4): 380-7.
4. Keyes PH. The infectious and transmissible nature of experimental dental caries. Arch Oral Biol. 1960; 1: 304-20.
5. Newbrun E. Cariology. 2nd ed. Baltimore: Williams & Wilkins; 1983.
6. Selwitz RH, Ismail AI, Pitts NB, Dental caries. Lancet. 2007; 369(9555): 51-9.

7. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res. 1994; 8(2): 263-71.

8. Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation--new insight. J Dent Res. 2006; 85(10): 878-87.

9. Tanner ACR, Kressirer CA, Rothmiller S, Johansson I, Chalmers NI. The Caries Microbiome: Implications for Reversing Dysbiosis. Adv Dent Res. 2018;29(1):78-85.

10. Xiao J, Klein MI, Delahunty CM, Yates III JR, Lu B, Heydorn A, et al. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog. 2012; 8(4): e1002623.

11. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res. 2013; 92(12): 1065-73.

12. Liao S, Klein MI, Heim KP, Fan Y, Bitoun JP, Ahn SJ, et al. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. J Bacteriol. 2014; 196(13): 2355-66.

13. Klein MI, Hwang G, Santos PHS, Campanella OH, Koo H. *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol. 2015; 5: 10.

14. Castillo Pedraza MC, Novais TF, Faustoferri RC, Quivey RG Jr, Terekhov A, Hamaker BR, et al. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of *Streptococcus mutans* biofilms. Biofouling. 2017; 33(9): 722-40.

15. Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology 2008; 154: 3247-55.

16. Bowen WH, Koo H. Biology of *Streptococcus mutans* derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011; 45(1): 69-86.

17. Florez Salamanca EJ, Klein MI. Extracellular matrix influence in *Streptococcus mutans* gene expression in a cariogenic biofilm. Mol Oral Microbiol. 2018; 33(2): 181-93.

18. Castillo Pedraza MC, Rosalen PL, de Castilho ARF, Freires IA, de Sales Leite L, Faustoferri RC, et al. Inactivation of *Streptococcus mutans* genes *lytST* and *dltAD* impairs its pathogenicity in vivo. J Oral Microbiol. 2019; 11(1): 1607505.

19. Mattos-Graner RO, Klein MI, Smith DJ. Lessons Learned from Clinical Studies: Roles of Mutans Streptococci in the Pathogenesis of Dental Caries. Curr. Oral Health Rep. 2014; 1: 70–78.

20. ten Cate JM. Novel anticaries and remineralizing agents: prospects for the future. J Dent Res. 2012; 91(9): 813-5.

21. Frazão P, Narvai PC. Cobertura e vigilância da fluoretação da água no Brasil: municípios com mais de 50 mil habitantes. 1a. ed. São Paulo: Universidade de São Paulo. Faculdade de Saúde Pública, 2017. 202p.

22. Griffin SO, Griffin PM, Swann JL, Zlobin N. New coronal caries in older adults: implications for prevention. J Dent Res. 2005; 84: 715-20.

23. Clarkson JJ, McLoughlin J. Role of fluoride in oral health promotion. Int Dent J. 2000; 50(3): 119-28.

24. Marquis RE, Clock SA, Mota-Meira M. Fluoride and organic weak acids as modulators of microbial physiology. FEMS Microbiol Rev 2003, 760: 1-18.

25. Gregoire S, Singh AP, Vorsa N, Koo H. Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. J Appl Microbiol. 2007; 103(5): 1960-8.

26. Jeon JG, Klein MI, Xiao J, Gregoire S, Rosalen PL, Koo H. Influences of naturally occurring agents in combination with fluoride on gene expression and structural organization of *Streptococcus mutans* in biofilms. BMC Microbiol. 2009; 9: 228.

27. Falsetta ML, Klein MI, Lemos JA, Silva BB, Agidi S, Scott-Anne KK, Koo H. Novel antibiofilm chemotherapy targets exopolysaccharide synthesis and stress tolerance in *Streptococcus mutans* to modulate virulence expression in vivo. Antimicrob Agents Chemother. 2012; 56(12): 6201-11.

28. Richter SG, Elli D, Kim HK, Hendrickx AP, Sorg JA, Schneewind O, et al. Small molecule inhibitor of lipoteichoic acid synthesis is an antibiotic for Gram-positive bacteria. Proc Natl Acad Sci U S A. 2013; 110(9): 3531-6.

29. Paganelli FL, van de Kamer T, Brouwer EC, Leavis HL, Woodford N, Bonten MJ, et al. Lipoteichoic acid synthesis inhibition in combination with antibiotics abrogates growth of multidrug-resistant *Enterococcus faecium*. Int J Antimicrob Agents. 2017; 49(3): 355-63.

30. Marinho VC, Higgins JP, Logan S, Sheiham A. Systematic review of controlled trials on the effectiveness of fluoride gels for the prevention of dental caries in children. J Dent Educ. 2003; 67(4): 448-58.

31. Zero DT. Dentifrices, mouthwashes, and remineralization/caries arrestment strategies. BMC Oral Health. 2006; 6 Suppl 1:S9.

32. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. CLSI document M100-S25. Wayne, PA. 2015. http://file.qums.ac.ir/repository/mmrc/CLSI2015.pdf

33. Saputo S, Faustoferri RC, Quivey RG Jr. A drug repositioning approach reveals that *Streptococcus mutans* is susceptible to a diverse range of established antimicrobials and nonantibiotics. Antimicrob Agents Chemother. 2017; 62(1): e01674-17.

34. Lemos JA, Abranches J, Koo H, Marquis RE, Burne RA. Protocols to study the physiology of oral biofilms. Methods Mol Biol. 2010; 666: 87-102.

35. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956; 28: 350–6.

36. Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, et al. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. Proc Natl Acad Sci U S A. 2007; 104(19): 8113-8.

37. Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilms development. Appl Environ Microbiol. 2009; 75(3): 837-41.

38. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology. 2000; 146(10): 2395-407.

39. Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AH, et al. Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One. 2010; 5(10): e13478.

40. Klein MI, Xiao J, Lu B, Delahunty CM, Yates III JR, Koo H. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. PLoS One. 2012; 7(9): e45795.

41. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55(4): 611-22.

42. Cury JA, Koo H. Extraction and purification of total RNA from *Streptococcus mutans* biofilms. Anal Biochem. 2007; 365(2): 208-14.

43. Yin JL, Shackel NA, Zekry A, et al. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol Cell Biol. 2001; 79(3): 213-21.

44. Castilho RM, Squarize CH, Leelahavanichkul K, Zheng Y, Bugge T, Gutkind JS. Rac1 is required for epithelial stem cell function during dermal and oral mucosal wound healing but not for tissue homeostasis in mice. PLoS One. 2010; 5(5): e10503.

45. International Organization for Standardization. Biological evaluation of medical devices. Part 5: Tests for cytotoxicity: *in vitro* methods, 3st ed, ISO 10993-5, 2009.

46. Jeon JG, Rosalen PL, Falsetta ML, Koo H. Natural products in caries research: current (limited) knowledge, challenges and future perspective. Caries research. 2011; 45(3): 243-63.

47. Vacca-Smith AM, Bowen WH. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. Arch Oral Biol. 1998; 43(2): 103-10.

48. Cross SE, Kreth J, Zhu L, Sullivan R, Shi W, Qi F, et al. Nanomechanical properties of glucans and associated cell-surface adhesion of *Streptococcus mutans* probed by atomic force microscopy under in situ conditions. Microbiology. 2007; 153: 3124-32.

49. Ahn SJ, Rice KC, Oleas J, Bayles KW, Burne RA. The *Streptococcus mutans* Cid and Lrg systems modulate. virulence traits in response to multiple environmental signals. Microbiology. 2010; 156(10): 3136-47.

50. Perry JA, Cvitkovitch DG, Lévesque CM. Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA. FEMS Microbiol Lett. 2009; 299(2): 261-6.

51. Kim M, Jeon J, Kim J. *Streptococcus mutans* extracellular DNA levels depend on the number of bacteria in a biofilm. Sci Rep. 2018; 8(1): 13313.

52. Kim M. Characterization of the effects of *Streptococcus mutans* growth conditions on eDNA release. 32nd Asia Pacific Dental and Oral Health Congress. July 23-24, 2018 Sydney, Australia. https://www.longdom.org/proceedings/characterization-of-the-effects-of-streptococcus-mutans-growth-conditions-on-edna-release-43070.html.

53. Neuhaus FC, Baddiley J. A continuum um of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev. 2003; 67: 686-723.

54. May JJ, Finking R, Wiegeshoff F, Weber TT, Bandur N, Koert U, et al. Inhibition of the D-alanine:D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. FEBS J. 2005; 272(12): 2993-3003.

55. Mazda Y, Kawada-Matsuo M, Kanbara K, Oogai Y, Shibata Y, Yamashita Y, et al. Association of CiaRH with resistance of *Streptococcus mutans* to antimicrobial peptides in biofilms. Mol Oral Microbiol. 2012; 27(2): 124-35.

56. Nilsson M, Rybtke M, Givskov M, Hoiby N, Twetman S, Tolker-Nielsen T. The *dlt* genes play a role in antimicrobial tolerance of *Streptococcus mutans* biofilms. Int J Antimicrob Agents. 2016; 48(3): 298-304.

57. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010; 8(9): 623-33.

58. Bowen WH, Hewitt MJ. Effect of fluoride on extracellular polysaccharide production by *Streptococcus mutans*. J Dent Res. 1974; 53(3): 627-29.

59. Guo L, Hu W, He X, Lux R, McLean J, Shi W. Investigating acid production by *Streptococcus mutans* with a surface-displayed pH-sensitive green fluorescent protein. PLoS One 2013; 8(2): e57182.

FIGURES

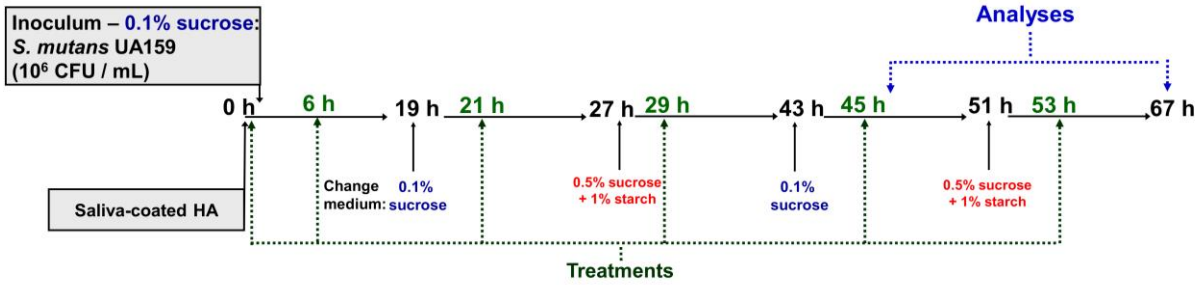

Figure 1. Experimental design for twice-daily topical treatments using a saliva-coated hydroxyapatite biofilm model.

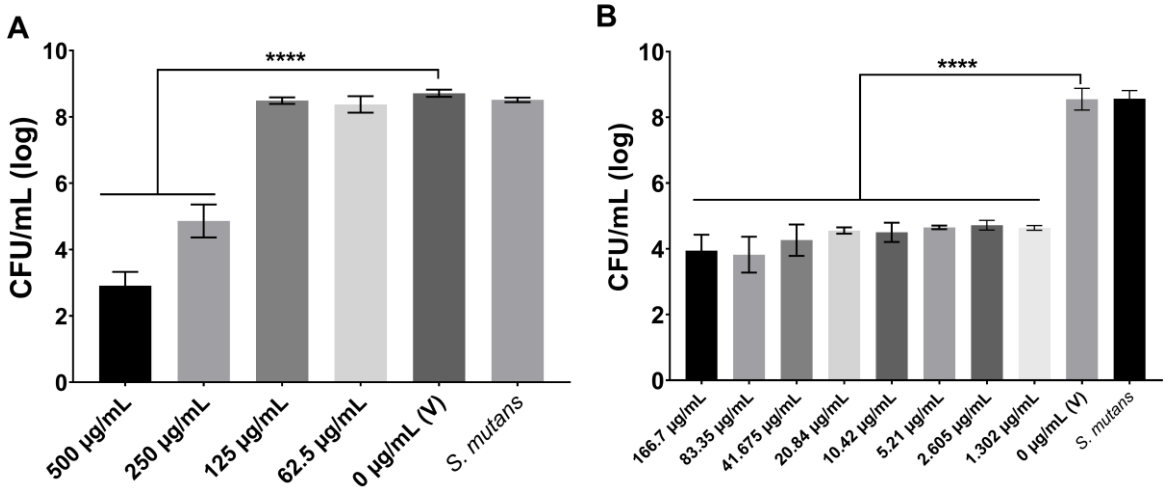

Figure 2. Antimicrobial activity of myricetin (A) and compound 1771 (B). The *S. mutans* viable population data are shown as average, and error bars correspond to the standard deviation (n=12). V: Vehicle; for myricetin, the vehicle was 7% ethanol in 1xPBS (pH 7.2); while for compound 1771, it was 7% ethanol and 1.25% DMSO. S. *mutans*: growth control culture. **** denotes a statistically significant difference between the vehicle and the concentrations tested. ($p<0.0001$; one-way ANOVA test, followed by Dunnett's multiple comparisons test).

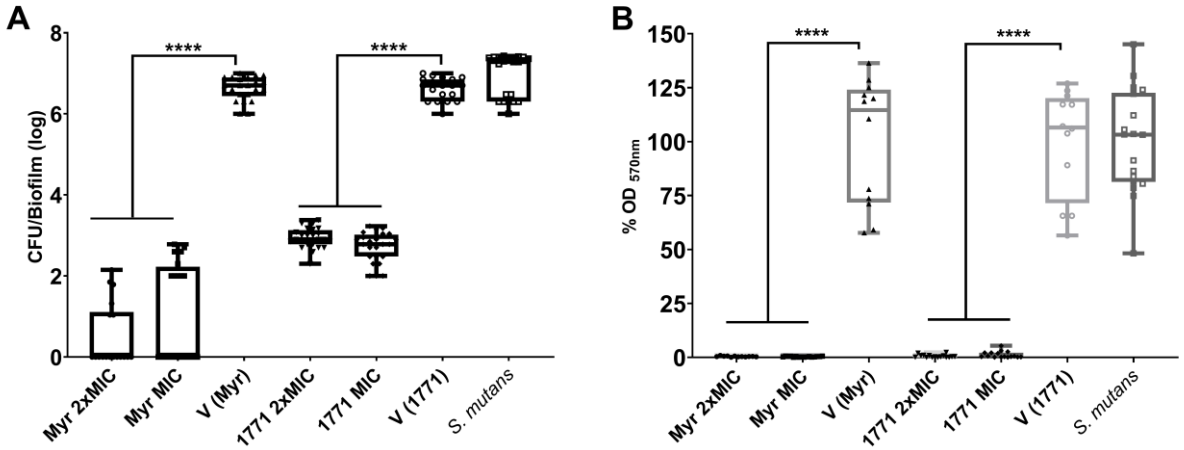

Figure 3. Antibiofilm activity of myricetin and compound 1771 tested at concentrations of MIC and 2xMIC using the polystyrene microplate model. The *S. mutans* viable population (A) and biomass (B) data are shown as the median and interquartile range (n=12). The two concentrations of the two agents were able to inhibit both viable population and biomass. *S. mutans*: growth control group. **** denotes the statistical difference between treatments and V ($p<0.0001$; Kruskal-Wallis test, followed by Dunn's post-test). Myr: myricetin. 1771: compound 1771. V: vehicle used for each compound.

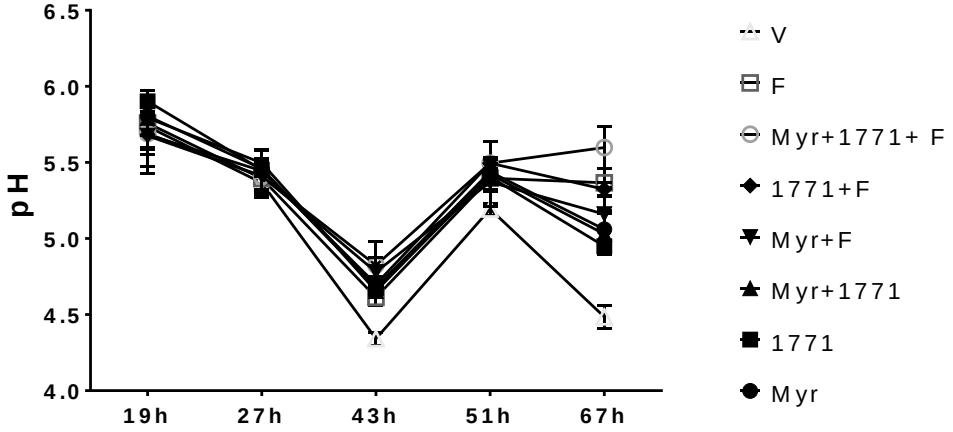

Figure 4. pH of spent culture media from topically treated biofilms at distinct developmental phases. The spent biofilm culture media were evaluated at 19, 27, 43, 51, and 67 h. At 19 h, the culture medium treated with compound 1771 showed a higher pH value when compared to Myr+F, 1771+F, Myr+1771, F and V. At 27 h, there was no significant difference between treatments. The biofilm culture medium with vehicle

showed lower pH at 43, 51, and 67 h. The medium from Myr+1771+F at 43 h had the highest pH value when compared to Myr+1771 and F and at 67 h had the highest pH value ($p<0.05$, one-way ANOVA, followed by Tukey's test). The data represented are the means, and the error bars correspond to the standard deviation.

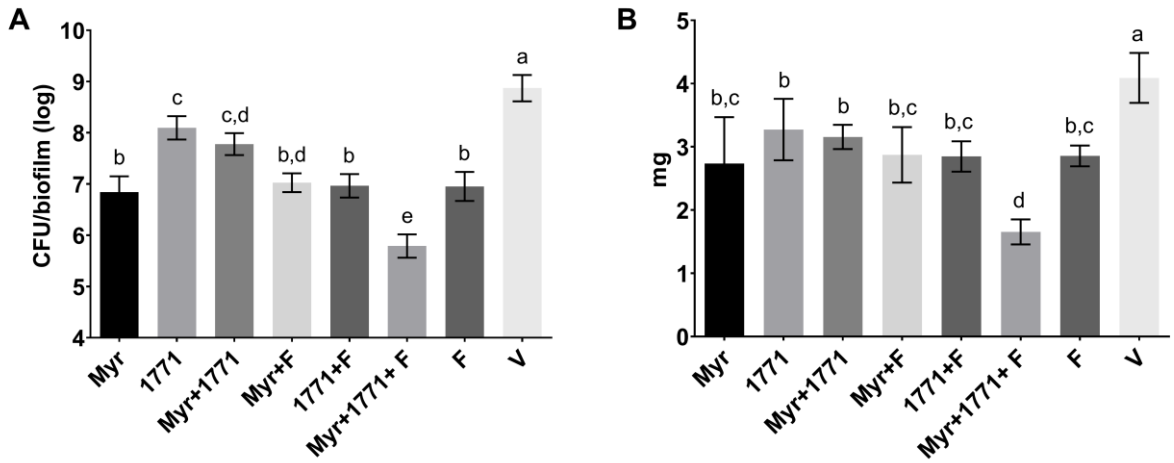

Figure 5. *S. mutans* viable counts (A) and insoluble biomass (dry-weight) (B) of topically treated biofilms. At 67 h, the vehicle yielded the highest values of CFU/biofilm and biomass, while the lowest values were found for the combination of Myr+1771+F. Bars with the same letters indicate no statistical difference between the different treatments in each graph ($p>0.05$; one-way ANOVA, followed by Tukey's test). The data represented are the means, and the error bars correspond to the standard deviation.

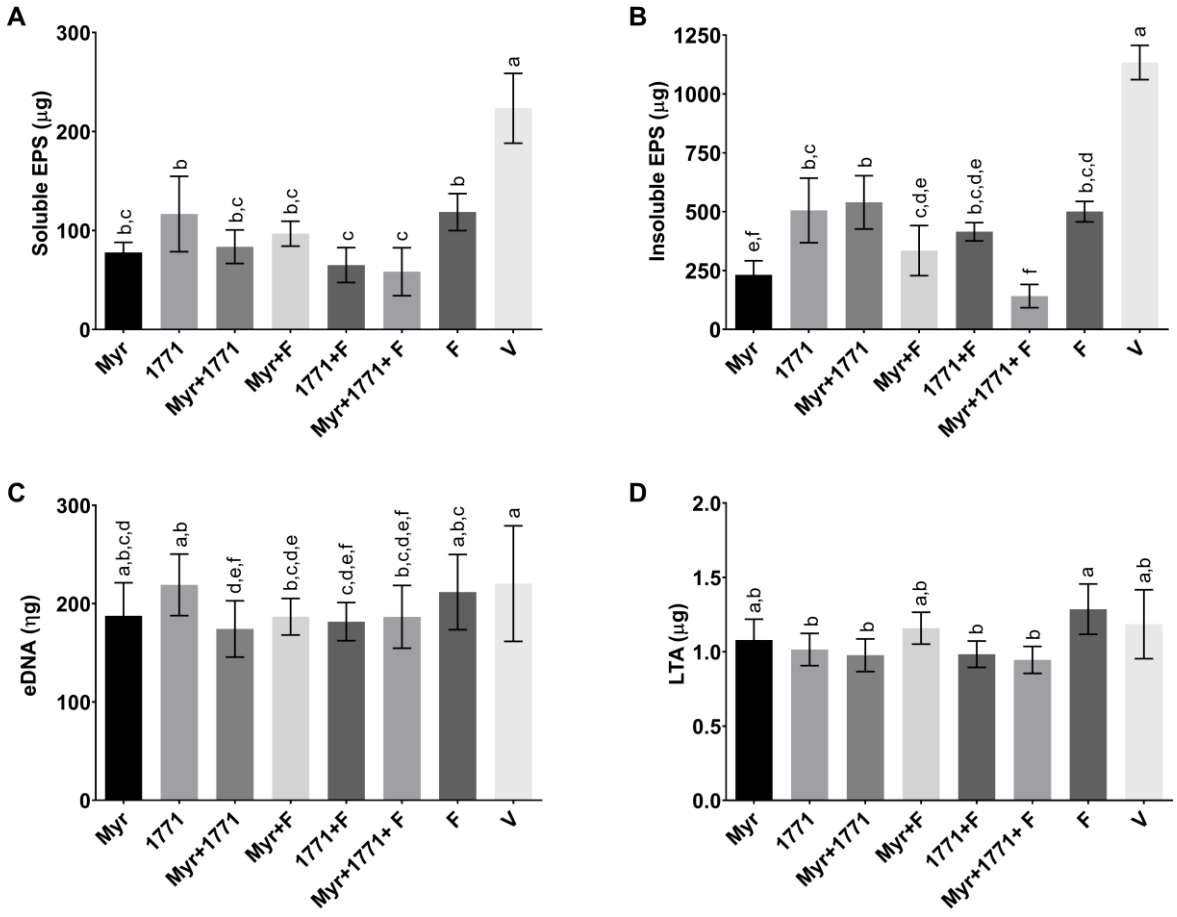

Figure 6. Modulation of the extracellular matrix components of the treated biofilms. The graphs exhibit the content of water-soluble EPS (A), water-insoluble EPS (B), eDNA (C), and LTA (D) in the extracellular matrix. Bars with the same letters indicate no statistical difference between the different treatments in each graph ($p>0.05$; one-way ANOVA, followed by Tukey's test). The data represented are the means, and the error bars correspond to the standard deviation.

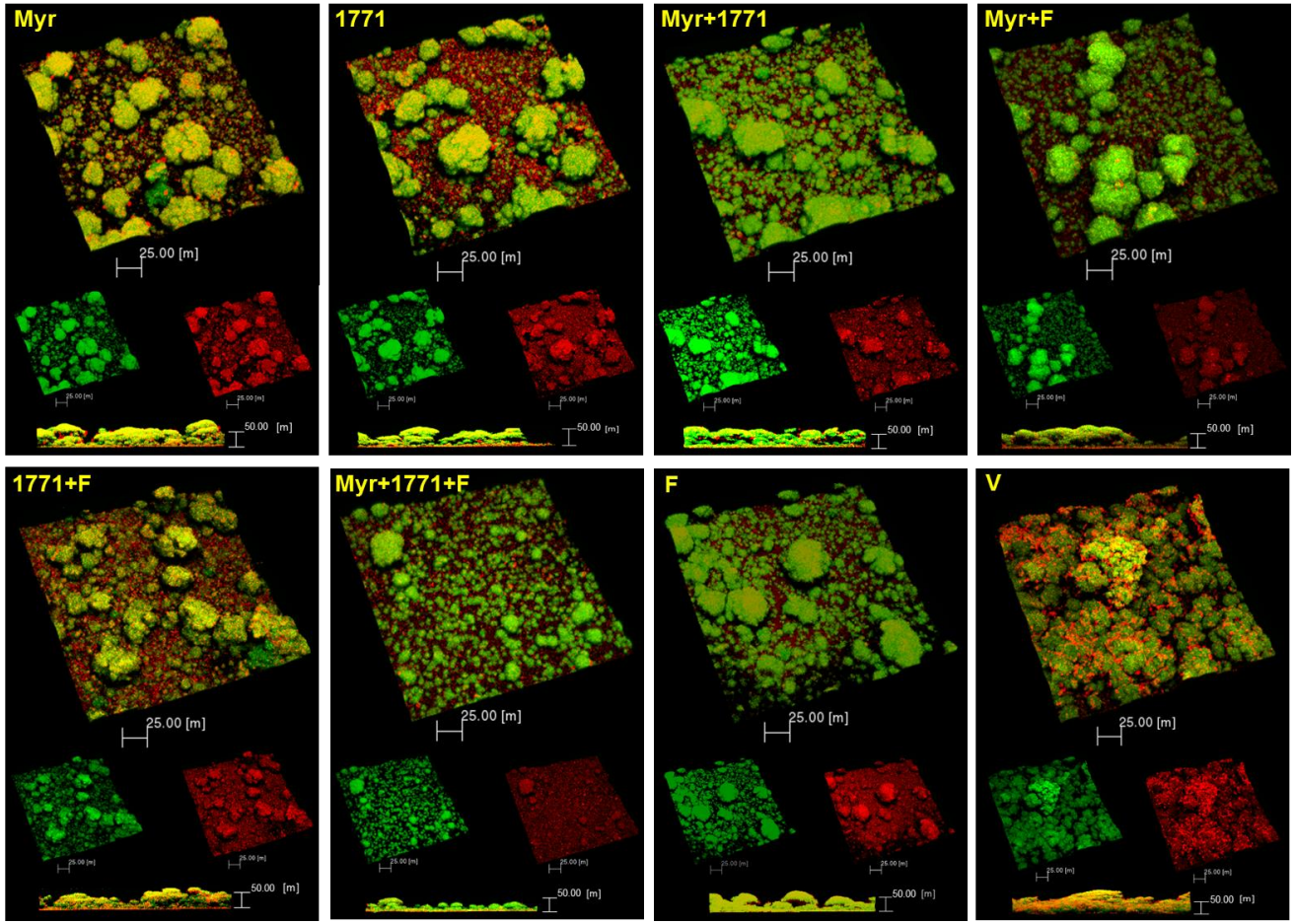

Figure 7. 3D architecture of the topically treated *S. mutans* biofilm. Representative images of 67 h-old biofilms are displayed in this image. The red color represents exopolysaccharides produced by *S. mutans* (Alexa Fluor 647) and the green color *S. mutans* cells (SYTO 9). The larger image in each set represents the overlapping images of the red and green channels, which are shown separately in a smaller size (scale bars of 25 μm). The bottom images are a cross-section of the biofilm with overlapping images of both channels (scale bars of 50 μm).

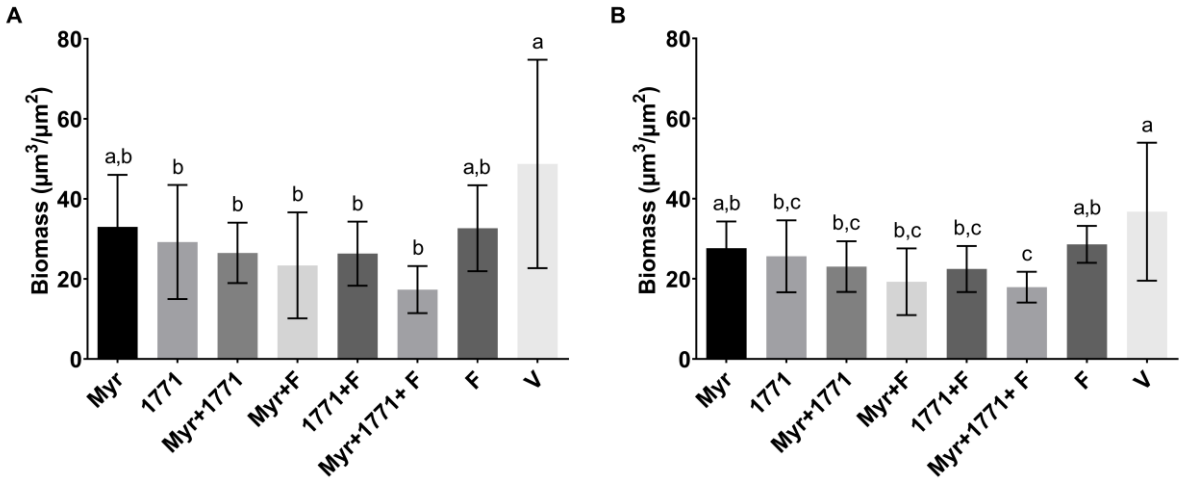

Figure 8. Biovolume of bacteria and EPS in topically treated biofilms. Biovolume is represented as biomass ($\mu m^3/\mu m^2$) of bacteria (A) and EPS (B). Bars with the same letters indicate no statistical difference between the different treatments in each graph ($p>0.05$; one-way ANOVA, followed by Tukey's test). The data represented are the means, and the error bars correspond to the standard deviation.

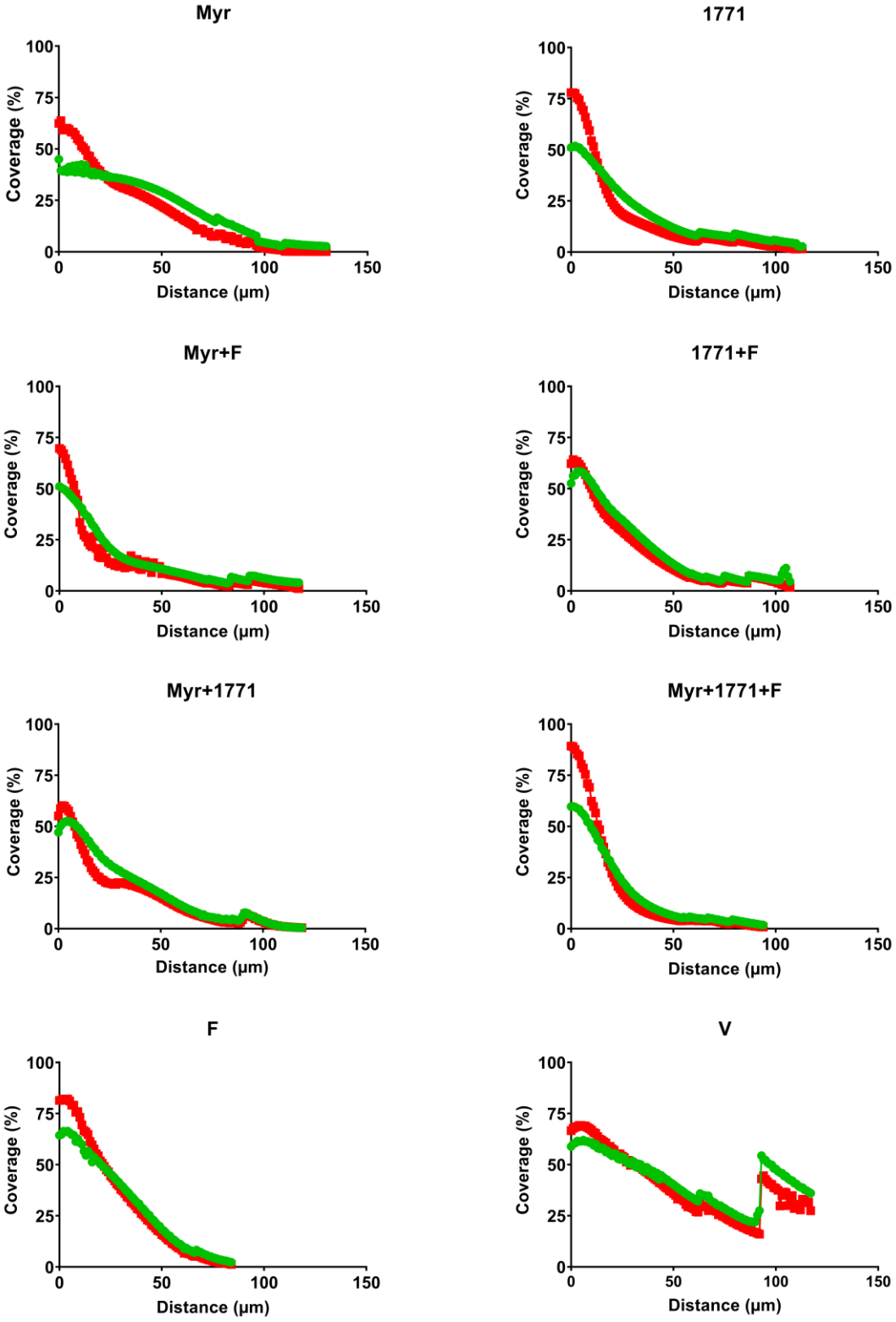

Figure 9. Profile of the distribution of bacteria and EPS in each of the topically treated biofilms. The data shown are the mean percentage coverage per area from the interface substratum/biofilm (hydroxyapatite disc) to the top (outer layer) of each biofilm at 67 h (n=12 images per biofilm).

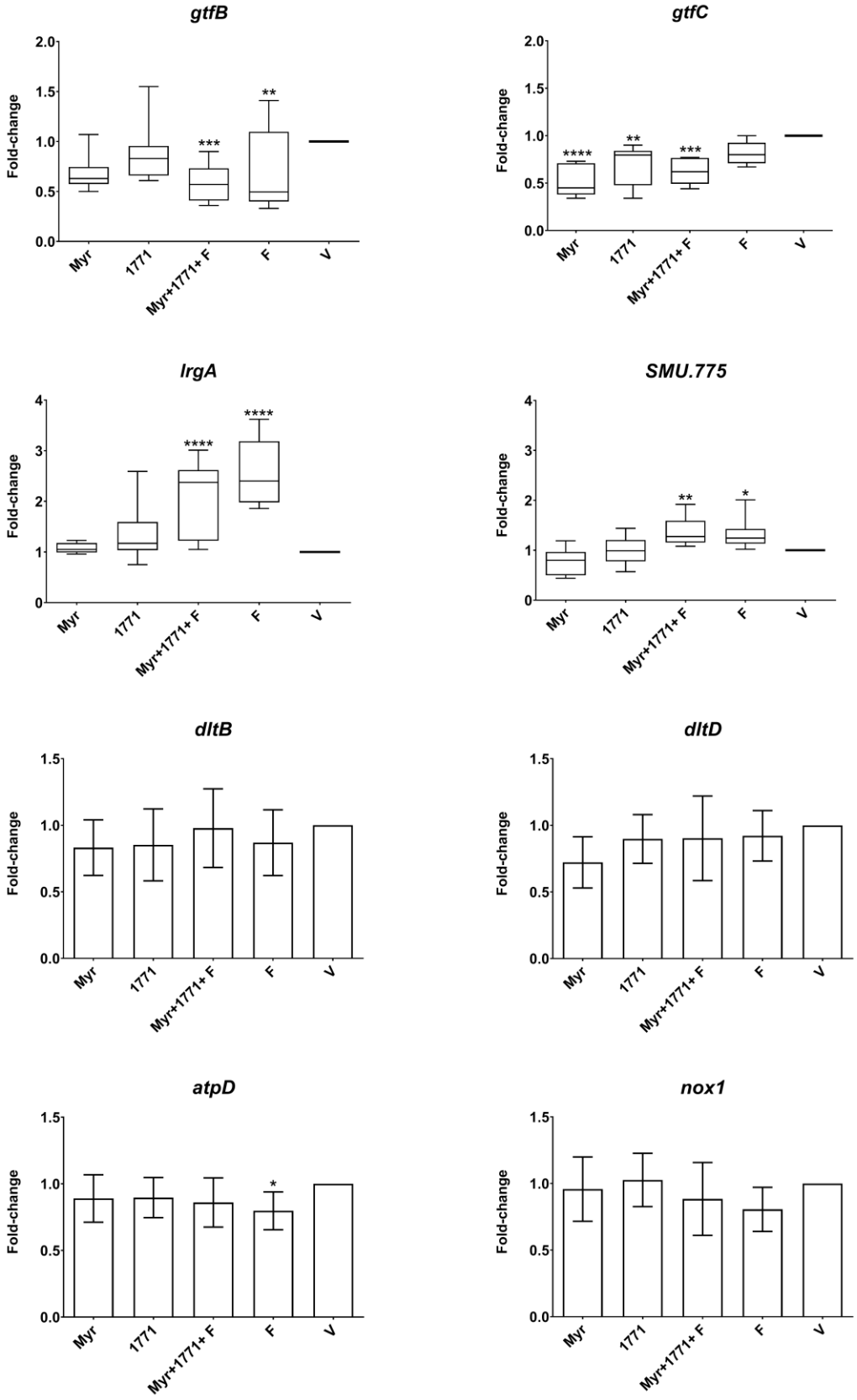

Figure 10. Gene expression profile of *S. mutans* biofilms after topical treatments. The fold-change is relative to vehicle-treated biofilms. Data presented are median and

interquartile range are shown for genes *gtfB*, *gtfC*, *IrgA*, and *SMU.775* (box plot graphs, Kruskal-Wallis test, followed by Dunn post-test), while mean and standard deviation for genes *dltB, dltD, atpD,* and *nox1* (bar graphs; one-way ANOVA, followed by Tukey's test), while median and interquartile range are shown for genes *gtfB*, *gtfC*, *IrgA*, and *SMU.775* (box plot graphs, Kruskal-Wallis test, followed by Dunn post-test). The asterisks depict significant statistical differences of treatments *versus* the vehicle, where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. The data were obtained from three experiments (with two cDNA per experiment), and the quantification of qPCR expression was performed in duplicate.

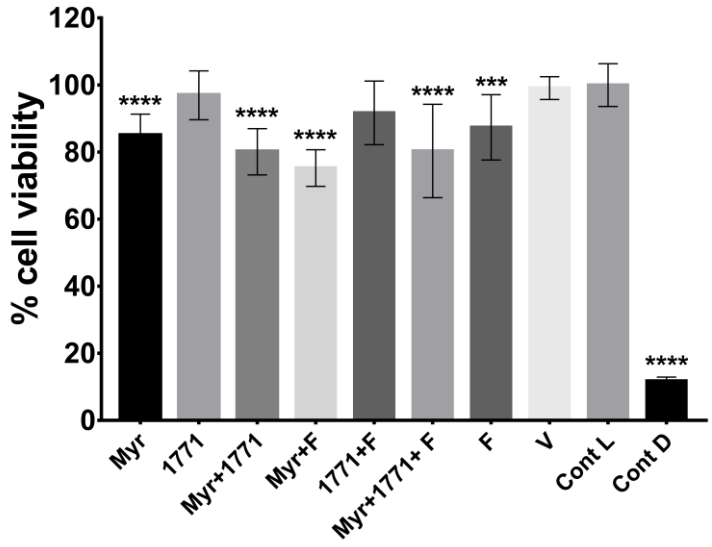

Figure 11. Cell viability of keratinocytes NOK-si after exposure to treatments. The data represented are the means, and the error bars correspond to the standard deviation "Cont L" indicates cell viability control and "Cont D", the cell death control. The percentage of cell viability was obtained considering the cell viability control as 100%. The asterisks denote a statistically significant difference of a specific extract *versus* cell viability control (Cont L), where ****$p<0.0001$ and ***$p=0.0005$ (one-way ANOVA, followed by Tukey's test).

4 DISCUSSÃO

A primeira publicação apresentada demonstrou que a deleção dos genes Δ*gtfB*, Δ*lytS*, Δ*lytT*, Δ*dltA* e Δ*dltD* afetam a virulência de *S. mutans* na superfície dentária (Ratos - Wistar) e na infecção sistêmica (*G. mellonella*)[52]. Este comportamento de *S. mutans* pode ser atribuído pela ausência dos produtos codificados pelos genes, essenciais na construção de uma matriz altamente coesiva[24].

As exoenzimas Gtfs de *S. mutans* favorecem a síntese de EPS na matriz quando a sacarose e o amido estão presentes na dieta[19]. A síntese e a organização tridimensional de EPS são fatores determinantes na ocorrência da cárie[29]. Isso pode ser visualizado neste estudo, onde a deleção de Δ*gtfB* ocasionou menor número de lesões cariosas sobre as superfícies lisa e sulcal dos dentes dos ratos. Portanto, o gene *gtfB* não só é determinante na construção da matriz extracelular, mas também é relacionado ao desenvolvimento e severidade de lesões cariosas[52].

Os genes *lytS* e *dltD* também estão envolvidos na cariogenicidade de *S. mutans;* pois a deleção desses genes ocasionou menores números de lesões de esmalte e de dentina em ratos (*vs*. a cepa parental UA159 com todos os genes). A interrupção do sistema de dois componentes *lytST* afeta a expressão de *lrgAB* em *S. mutans*, interferindo na regulação da autólise e assim, na liberação de eDNA[39,40]. Adicionalmente, os genes *dltABCD* são necessários para a adição de resíduos de D-alanina durante o metabolismo de LTA[45]; esses resíduos afetam os processos de adesão à superfície da hidroxiapatita e a formação de biofilmes[48,53]. A interação de EPS, eDNA e LTA favorece a espécie *S. mutans* na colonização e na formação de biofilme com uma matriz altamente coesiva[24,35,38]. Portanto, a falta de genes funcionais relacionados ao metabolismo de EPS, eDNA e LTA prejudica a cariogenicidade de *S. mutans*[53].

Adicionalmente, demonstrou-se que a deleção dos genes *gtfB, lytS, lytT, dltA* e *dltD* causou menores taxas de mortalidade larval de *G. mellonella*[52]; assim, esses genes poderiam afetar a virulência de *S. mutans* para causar doenças extraorais. No entanto, a cepa Δ*gtfB* apresentou maior capacidade de matar mais larvas quando comparada às demais cepas com deleção. *S. mutans* pode estar se defendendo dos mecanismos de proteção da larva (por exemplo, produção de espécie reativas de oxigênio) pela tolerância ao estresse oxidativo conferida pelos genes *lytST* e *dltAD*[52].

Assim, a maior sobrevida das larvas pode estar relacionada à menor tolerância ao estresse oxidativo após a exposição ao H_2O_2.

Portanto, os genes *lytST* e *dltAD* contribuem na virulência de *S. mutans* para desenvolver cárie dentária e em infecção sistêmica. Esse resultado forneceu subsídios para testar terapias que inibam os produtos codificados por esses genes, para minimizar ou evitar os danos causados por eles e seus produtos nas superfícies dos dentes e prevenir a cárie dentária.

Deste modo, na segunda publicação foi testado o composto 1771 (inibe o metabolismo de LTA em bactérias Gram-positivas), a miricetina (flavonóide que inibe a expressão de *gtfBC* e a síntese de EPS) e o flúor (pilar da prevenção da cárie). A aplicação tópica do composto 1771 e da miricetina isoladamente impede o desenvolvimento de biofilmes de *S. mutans*. No entanto, o efeito desses agentes bioativos foi mais pronunciado quando foram associados ao flúor (Publicação 2).

A terapia combinada (Myr+1771+F) reduziu pronunciadamente os dados de população de *S. mutans*, biomassa, EPS solúveis e insolúveis. Ainda, mesmo sem afetar marcadamente LTA e eDNA, as quantidades dessas duas biomoléculas presentes na matriz foram necessárias para contribuir com a construção e a organização 3D dos biofilmes. O conteúdo de LTA na matriz dos biofilmes tratados foi significativamente reduzido apenas nos quatro tratamentos contendo o composto 1771 (1771, Myr+1771, 1771+F e Myr+1771+F) (Publicação 2). Uma pequena redução de LTA também pode estar contribuindo para alteração na arquitetura do biofilme, população viável de *S. mutans*, biomassa e EPS na matriz.

Entretanto, a expressão de *lrgA* e *SMU.775* foi maior nos biofilmes tratados com a combinação de Myr+1771+F e F sozinho; assim, esse comportamento pode estar associado ao flúor. O gene *lrgA* está envolvido no processo de remodelação e autólise da parede celular de *S. mutans* e pode originar eDNA na matriz[39,54]. Portanto, um aumento na expressão de *lrgA* pode indicar que esses tratamentos desencadeiam a remodelação da parede celular e / ou a autólise. Além disso, eDNA pode ser secretado ativamente a partir de células vivas de *S. mutans* através de vias metabólicas adicionais envolvidas na secreção de proteínas e componentes para inserção de proteínas em membranas (i.e., Ffh, YidC1 e YidC2)[38]. No entanto, o eDNA poderia ser liberado, mas poderia não ser incorporado na matriz se os tratamentos estiverem impedindo a síntese e / ou ligação de EPS. Consequentemente, o eDNA como componente solúvel na matriz pode estar no meio de cultura "velho"[55,56], ou ser

consumido como nutriente durante os períodos com baixa concentração de carboidratos no meio e não como parte da estrutura dos biofilmes, afetando a arquitetura 3D dos biofilmes tratados (*vs*. veículo) (Publicação 2).

SMU.775 é um gene hipotético com homologia à sintase de LTA em *S. aureus* e, portanto, poderia estar envolvido no metabolismo da LTA[35]. Adicionalmente, não houve diferenças significativas na expressão dos genes *dltB* e *dltD* entre os tratamentos e veículo (Publicação 2). Vários estudos demonstraram que a interferência no processo de D-alanização de LTA afeta a carga superficial das células e a susceptibilidade das células a antimicrobianos[51,57,58]. No entanto, é possível que o composto 1771 tenha interferido no metabolismo do LTA e afetado a carga e / ou composição da superfície celular, interferindo assim na forma como EPS se ligaria às células e mediaria a formação de microcolônias e distribuição de EPS na estrutura 3D dos biofilmes tratados (Publicação 2).

A expressão de *nox1* relacionada à resposta ao estresse oxidativo foi semelhante entre os tratamentos testados e o veículo. No entanto, o F diminuiu ligeiramente a expressão de *atpD*, envolvido na tolerância ao ácido de *S. mutans*. Foi demonstrado que o flúor afeta a atividade glicolítica em células bacterianas e inibe as enzimas intracelulares quando interfere com a permeabilidade da membrana aos prótons[59], afetando a tolerância de *S. mutans* aos microambientes acídicos dentro de um biofilme[60,61]. A miricetina também contribui na diminuição da aciduricidade, mediante a inibição da atividade glicolítica pela alteração da permeabilidade da membrana[61]. No entanto, neste estudo, a miricetina e o composto 1771 isolados ou combinados com flúor, não interferiram na expressão de *atpD*; isso pode ter ocorrido com base nas concentrações utilizadas, no tempo de exposição e / ou na fase de desenvolvimento do biofilme avaliada. No entanto, foi demonstrado que os EPS insolúveis são um fator essencial que intermedeia a acidogenicidade e a aciduricidade[62]. Desse modo, a combinação aqui testada influenciaria não apenas a composição da matriz, contagem de células viáveis, mas também como as células do biofilme respondem a um ambiente ácido durante o crescimento do biofilme.

O tratamento com a combinação Myr+1771+F mostrou menor expressão dos genes *gtfB* e *gtfC* (*versus* o veículo). No entanto, a expressão de *gtfB* também foi reprimida por flúor sozinho e a expressão de *gtfC* foi regulada negativamente por Myr e 1771 isoladamente. O comportamento desses genes, está relacionada com as diferenças na localização das exoenzimas GtfB e GtfC e a sua produção de EPS, o

que pode explicar a redução nas quantidades de EPS solúveis e insolúveis, biomassa, capacidade de sobrevivência de *S. mutans* no biofilme e o padrão distinto de estrutura 3D encontrado para biofilmes tratados (Publicação 2).

A estratégia com a combinação Myr+1771+F diminuiu a morfologia das microcolônias dentro do biofilme. Microcolônias menores apresentam menos formação de micronichos ácidos, interferindo na adesão e na estrutura do biofilme, evitando a desmineralização dentária[25]. Assim, o uso de miricetina e composto 1771 combinado com flúor pode ser uma boa estratégia para redução da patogenicidade do biofilme dental. A utilização da miricetina e do composto 1771 não se destina a eliminar o flúor, mas sim, melhorar a eficácia cariostática deste produto.

Embora o modelo de biofilme de *S. mutans* empregado na publicação 2 não imite a interação biológica de microrganismos dentro de uma placa dentária cariogênica, o mesmo mostra que os agentes testados interferiram nas propriedades críticas do biofilme. Estudos futuros são necessários para avaliar se a terapia de Myr+1771+F afeta a microbiota oral complexa e previne lesões cariosas in vivo e se o aumento da concentração de agentes e / ou do tempo de exposição / retenção, pode tornar a estratégia eficaz em um cenário clínico.

Assim, a ausência de genes importantes para a biologia de *S. mutans* e a construção da matriz do biofilme diminui a virulência em modelos de cárie e infeção sistêmica (Publicação 1); sobretudo, a modulação desses genes por terapia direcionada altera a composição da matriz do biofilme e diminui o potencial cariogênico de *S. mutans* (Publicação 2).

5 CONCLUSÃO

Ausência funcional dos genes *gtfB*, *lyST* e *dltAD* de *S. mutans* diminui a cariogenicidade de biofilmes formados sobre a superfície dentária e a virulência em infecções sistêmicas; ainda estratégia com miricetina e composto 1771 modula os produtos destes genes, tornando-a uma terapia antibiofilme promissora. Portanto, as conclusões especificas desta pesquisa são:

- A inativação do gene *gtfB, lytS* e *dltD* desencadeia menor quantidade de lesões cariosas em modelo animal em superfícies lisas e sulcos, pela ausência dos seus produtos que favorecem a formação do biofilme cariogênico.
- A ausência dos genes *gtfB*, *lytST* e *dltAD* de *S. mutans* diminui a virulência desta espécie em infecção sistêmica in vivo (modelo incipiente - *Galleria mellonella*). A redução na infecção sistêmica pode ser em parte porque a ausência desses genes tornou as cepas com deleção (exceto ∆*gtfB*) mais suscetíveis ao estresse oxidativo, que faz parte do sistema imune inato de *G. mellonella*.
- A aplicação tópica do composto 1771 e da miricetina isoladamente impediu o acúmulo de biofilme de *S. mutans* (in vitro). No entanto, o efeito é mais pronunciado quando foi combinado o composto 1771, a miricetina e o flúor. A estratégia combinada afeta a quantidade dos componentes na matriz, a arquitetura 3D dos biofilmes e a expressão dos genes que codificam exoenzimas que sintetizam EPS.

REFERÊNCIAS*

1. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet. 2007; 369(9555): 51-9.

2. Hicks J, Garcia-Godoy F, Flaitz C. Biological factor in dental caries: role of saliva and dental plaque in dynamic process of demineralization and remineralization (Part 1). J Clin Pediatr Dent. 2003; 28(1): 47-52.

3. Hicks J, Garcia-Godoy F, Flaitz C. Biological factor in dental caries: enamel structure and caries process in the dynamic process of demineralization and remineralization (Part 2). J Clin Pediatr Dent. 2004; 28(2): 119-24.

4. Hicks J, Garcia-Godoy F, Flaitz C. Biological factor in dental caries: role of remineralization and fluoride in the dynamic process of demineralization and remineralization (Part 3). J Clin Pediatr Dent. 2004; 28(3): 203-14.

5. Keyes PH. The infectious and transmissible nature of experimental dental caries. Arch Oral Biol. 1960; 1: 304-20.

6. Newbrun E. Cariology. 2nd ed. Baltimore: Williams & Wilkins; 1983.

7. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology. 2003; 149(2): 279-94.

8. Manji F, Fejerskov O. Dental caries in developing countries in relationship to the apropiate use of fluorides. J Dent Res. 1990; 69(2): 733-41.

9. Marcenes W, Kassebaum NJ, Bernabé E, Flaxman A, Naghavi M, Lopez A et al. Global burden of oral conditions in 1990-2010: a systematic analysis. J Dent Res. 2013; 92(7): 592-7.

10. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of untreated caries: a systematic review and metaregression. J Dent Res. 2015; 94(5): 650-8.

11. Pitts NB, Zero DT, Marsh PD, Ekstrand K, Weintraub JA, Ramos-Gomez F et al. Dental caries. Nat Rev Dis Primers. 2017; 3: 17030.

12. Holmes RD. Tooth brushing frequency and risk of new carious lesions. Evid Based Dent. 2016; 17(4): 98-9.

13. GBD 2016 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the GBD study 2016. Lancet. 2017; 390(10100): 1211-59.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

14. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA et al. Bacterial diversity in human subgingival plaque. J Bacteriol. 2001; 183(12): 3770–83.

15. Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation-new insight. J Dent Res. 2006; 85(10): 878-87.

16. Klein MI, Hwang G, Santos PHS, Campanella OH, Koo H. *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol. 2015; 5(10): 1-10.

17. Gronroos L, Saarela M, Matto J, Tanner-Salo U, Vuorela A, Alaluusa S. Mutacin production by *Streptococcus mutans* may promote transmission of bacteria from mother to child. Infect Immun. 1998; 66(6): 2595-600.

18. Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology. 2008; 154(11): 3247–55.

19. Bowen WH, Koo H. Biology of *Streptococcus mutans* derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011; 45(1): 69-86.

20. Takahashi N. Oral microbiome metabolism: from "Who are tthey?" to "What are they doing?". J Dent Res. 2015; 94(12): 1628-37.

21. Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. Infect Immun. 1993; 61(9): 3811-7.

22. Ribeiro CC, Tabchoury CP, Del Bel Cury AA, Tenuta LM, Rosalen PL, Cury JA. Effect of starch on the cariogenic potential of sucrose. Br J Nutr. 2005; 94(1): 44-50.

23. Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AH et al. Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One. 2010; 5(10): e13478.

24. Castillo Pedraza MC, Novais TF, Faustoferri RC, Quivey RG Jr, Terekhov A, Hamaker BR et al. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of *Streptococcus mutans* biofilms. Biofouling. 2017; 33(9): 722-40.

25. Xiao J, Klein MI, Falsetta ML, Delahunty CM, Yates III JR, Lu B et al. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathogens. 2012; 8(4): e1002623.

26. Bowen WH, Schilling K, Giertsen E, Pearson S, Lee SF, Bleiweis A et al. Role of a cell surface associated protein in adherence and dental caries. Infect Immun. 1991; 59(12): 4606-9

27. Vacca-Smith AM, Bowen WH. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. Arch Oral Biol. 1998; 43(2): 103-10.

28. Gregoire S, Xiao J, Silva BB, Gonzalez I, Agidi PS, Klein MI et al. Role of glucosyltransferase B in the interactions of *Candida albicans* with *Streptococcus mutans* and experimental pellicle formed on hydroxyapatite surface. Appl Environ Microbiol. 2011; 77(18): 6357-67.

29. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res. 2013; 92(12): 1065-73.

30. Inaba H, Amano A. Roles of oral bacteria in cardiovascular diseases - from molecular mechanisms to clinical cases: implication of periodontal diseases in development of systemic diseases. J Pharmacol Sci. 2010; 113(2): 103-9.

31. Freires IA, Avilés-Reyes A, Kitten T, Simpson-Haidaris PJ, Swartz M, Knight PA et al. Heterologous expression of *Streptococcus mutans* Cnm in *Lactococcus lactis* promotes intracellular invasion, adhesion to human cardiac tissues and virulence. Virulence. 2017; 8(1): 18-29.

32. Ferro JM, Fonseca AC. Infective endocarditis. Handb Clin Neurol. 2014; 119: 75-91.

33. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010; 8(9): 623-33.

34. Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol. 2005; 13(1): 20-6.

35. Karatan E, Watnick P. Signals, regulatory networks, and materials that buid and break bacterial biofilms. Microbiol Mol Bio. 2009; 73: 310-47.

36. Schooling SR, Hubley A, Beveridge TJ. Interactions of DNA with biofilm-derived membrane vesicles. J Bacteriol. 2009; 191(13): 44097-102.

37. Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One. 2009; 4(6): e5822.

38. Liao S, Klein MI, Heim KP, Fan Y, Bitoun JP, Ahn SJ et al. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. J Bacteriol. 2014; 196(13): 2355-66.

39. Ahn SJ, Rice KC, Oleas J, Bayles KW, Burne RA. The *Streptococcus mutans* Cid and Lrg systems modulate. Virulence traits in response to multiple environmental signals. Microbiology. 2010; 156(10): 3136-47.

40. Ahn SJ, Qu MD, Roberts E, Burne RA, Rice KC. Identification of the *Streptococcus mutans* LytST two-component regulon reveals its contribution to oxidative stress tolerance. BMC Microbiology. 2012; 12: 1-12.

41. Kuramitsu HK, Wondrack L, McGuinness M. Interaction of *Streptococcus mutans* glucosyltransferases with teichoic acids. Infect Immun. 1980; 29(2): 376-82.

42. Rölla G, Oppermann RV, Bowen WH, Ciardi JE, Knox KW. High amounts of lipoteichoic acid in sucrose induced plaque in vivo. Caries Res. 1980; 14(4): 235-8.

43. Ellwood DC, Tempest DW. Effects of environment on bacterial wall content and composition. Adv Microb Physiol. 1972; 7: 83–116.

44. Brock JH, Reiter B. Chemical and biological properties of extracellular slime produced by *Staphylococcus aureus* grown in high-carbohydrate, high-salt medium. Infect Immun. 1976; 13(3): 653–60.

45. Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev. 2003; 67(4): 686-723.

46. Florez Salamanca EJ, Klein MI. Extracellular matrix influence in *Streptococcus mutans* gene expression in a cariogenic biofilm. Mol Oral Microbiol. 2018; 33(2): 181-93.

47. Tanzer JM, Freedman ML, Fitzgerald RJ. Virulence of mutants defective in glucosyltransferase, dextran mediated aggregation, or dextranase activity. In: Mergenhgen SE, Rosan B, editors. Molecular basis of oral microbial adhesion. Washington DC: American Society for Microbiology; 1985.

48. Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun. 2001; 69(5): 3423–6.

49. Gründling A, Schneewind O. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. Proc Natl Acad Sci USA. 2007; 104(20): 8478-83.

50. Ahn SJ, Rice KC. Understanding the *Streptococcus mutans* Cid/Lrg system through CidB function. Appl Environ Microbiol. 2016; 82(20): 6189-203.

51. Nilsson M, Rybtke M, Givskov M. The *dlt* genes play a role in antimicrobial tolerance of *Streptococcus mutans* biofilms. Int J Antimicrob Agents. 2016; 48(3): 298-304.

52. Castillo Pedraza MC, Rosalen PL, de Castilho ARF, Freires IA, de Sales Leite L, Faustoferri RC et al. Inactivation of *Streptococcus mutans* genes *lytST* and *dltAD* impairs its pathogenicity in vivo. J Oral Microbiol. 2019; 11(1): 1607505.

53. Spatafora GA, Sheets M, June R, Luyimbazi D, Howard K, Hulbert R et al. Regulated expression of the *Streptococcus mutans* dlt genes correlates with intracellular polysaccharide accumulation. J Bacteriol. 1999; 181(8): 2363-72.

54. Perry JA, Cvitkovitch DG, Lévesque CM. Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA. FEMS Microbiol Lett. 2009; 299(2): 261-6.

55. Kim M, Jeon J, Kim J. *Streptococcus mutans* extracellular DNA levels depend on the number of bacteria in a biofilm. Sci Rep. 2018; 8(1): 13313.

56. Kim M. Characterization of the effects of *Streptococcus mutans* growth conditions on eDNA release. Proceedings of the 32nd Asia Pacific Dental and Oral Health Congress; 2018 jul 23 – 24; Sydney, Australia. [acesso em 2019 jul 14]. Disponível em: https://www.longdom.org/proceedings/characterization-of-the-effects-of-streptococcus-mutans-growth-conditions-on-edna-release-43070.html.

57. May JJ, Finking R, Wiegeshoff F, Weber TT, Bandur N, Koert U et al. Inhibition of the D-alanine: D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. FEBS J. 2005; 272(12): 2993-3003.

58. Mazda Y, Kawada-Matsuo M, Kanbara K, Oogai Y, Shibata Y, Yamashita Y et al. Association of CiaRH with resistance of *Streptococcus mutans* to antimicrobial peptides in biofilms. Mol Oral Microbiol. 2012; 27(2): 124-35.

59. Bowen WH, Hewitt MJ. Effect of fluoride on extracellular polysaccharide production by *Streptococcus mutans*. J Dent Res. 1974; 53(3): 627-29.

60. Marquis RE, Clock SA, Mota-Meira M. Fluoride and organic weak acids as modulators of microbial physiology. FEMS Microbiol Rev 2003, 26(5): 1-18.

61. Jeon JG, Klein MI, Xiao J, Gregoire S, Rosalen PL, Koo H. Influences of naturally occurring agents in combination with fluoride on gene expression and structural organization of *Streptococcus mutans* in biofilms. BMC Microbiol. 2009; 9: 228.

62. Guo L, Hu W, He X, Lux R, McLean J, Shi W. Investigating acid production by *Streptococcus mutans* with a surface-displayed pH-sensitive green fluorescent protein. PLoS One 2013; 8(2): e57182.

ANEXO A

Publicações 1 e 2. Aprovação para inclusão na tese de publicação do periódico *Journal of Oral Microbiology* segundo RoMEO.

SHERPA/RoMEO

Home • Search • Journals • Publishers •

Advanced Search - Publisher copyright policies & self-archiving

English | |

One journal found when searched for: **j oral microbiol**

Journal:	Journal of Oral Microbiology [1] (ESSN: 2000-2297)
RoMEO:	This is a RoMEO **green** journal
Listed in:	DOAJ as an open access journal
Author's Pre-print:	✓ author **can** archive pre-print (ie pre-refereeing)
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Updated:	15-Feb-2017 - Suggest an update for this record
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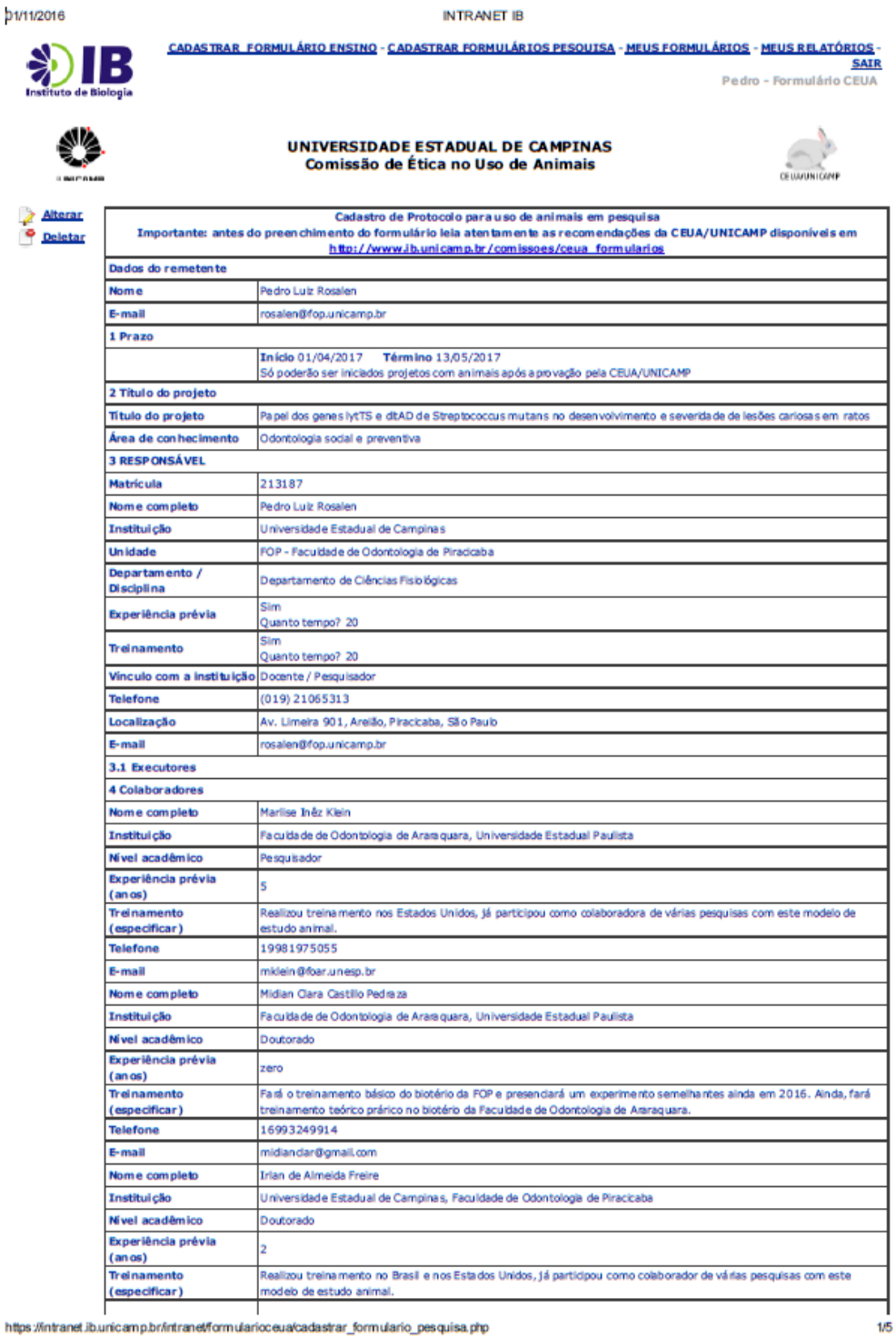
These summaries are for the journal's *default* policies, and changes or exceptions can often be negotiated by authors.
All information is correct to the best of our knowledge but should not be relied upon for legal advice.

ANEXO B

Aprovação do projeto pelo Comitê de Ética no Uso de Animais da Faculdade de Odontologia de Piracicaba – FOP/UNICAMP.



01/11/2016 INTRANET IB

Instituto de Biologia

CADASTRAR FORMULÁRIO ENSINO - CADASTRAR FORMULÁRIOS PESQUISA - MEUS FORMULÁRIOS - MEUS RELATÓRIOS - SAIR

Pedro - Formulário CEUA

UNIVERSIDADE ESTADUAL DE CAMPINAS
Comissão de Ética no Uso de Animais

CEUA/UNICAMP

Alterar
Deletar

Cadastro de Protocolo para uso de animais em pesquisa Importante: antes do preenchimento do formulário leia atentamente as recomendações da CEUA/UNICAMP disponíveis em http://www.ib.unicamp.br/comissoes/ceua_formularios	
Dados do remetente	
Nome	Pedro Luiz Rosalen
E-mail	rosalen@fop.unicamp.br
1 Prazo	
	Início 01/04/2017 **Término** 13/05/2017 Só poderão ser iniciados projetos com animais após aprovação pela CEUA/UNICAMP
2 Título do projeto	
Título do projeto	Papel dos genes lytTS e dltAD de Streptococcus mutans no desenvolvimento e severidade de lesões cariosas em ratos
Área de conhecimento	Odontologia social e preventiva
3 RESPONSÁVEL	
Matrícula	213187
Nome completo	Pedro Luiz Rosalen
Instituição	Universidade Estadual de Campinas
Unidade	FOP - Faculdade de Odontologia de Piracicaba
Departamento / Disciplina	Departamento de Ciências Fisiológicas
Experiência prévia	Sim Quanto tempo? 20
Treinamento	Sim Quanto tempo? 20
Vínculo com a instituição	Docente / Pesquisador
Telefone	(019) 21065313
Localização	Av. Limeira 901, Areião, Piracicaba, São Paulo
E-mail	rosalen@fop.unicamp.br
3.1 Executores	
4 Colaboradores	
Nome completo	Marilise Inêz Klein
Instituição	Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista
Nível acadêmico	Pesquisador
Experiência prévia (anos)	5
Treinamento (especificar)	Realizou treinamento nos Estados Unidos, já participou como colaboradora de várias pesquisas com este modelo de estudo animal.
Telefone	19981975055
E-mail	mklein@foar.unesp.br
Nome completo	Midian Clara Castillo Pedreza
Instituição	Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista
Nível acadêmico	Doutorado
Experiência prévia (anos)	zero
Treinamento (especificar)	Fará o treinamento básico do biotério da FOP e presenciará um experimento semelhantes ainda em 2016. Ainda, fará treinamento teórico prárico no biotério da Faculdade de Odontologia de Araraquara.
Telefone	16993249914
E-mail	midianclar@gmail.com
Nome completo	Irlan de Almeida Freire
Instituição	Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba
Nível acadêmico	Doutorado
Experiência prévia (anos)	2
Treinamento (especificar)	Realizou treinamento no Brasil e nos Estados Unidos, já participou como colaborador de várias pesquisas com este modelo de estudo animal.

https://intranet.ib.unicamp.br/intranet/formularioceua/cadastrar_formulario_pesquisa.php 1/5

ANEXO C

Aprovação para atividades em contenção com organismos geneticamente modificados e seus derivados pela Comissão Interna de Biossegurança da Faculdade de Odontologia de Piracicaba - FOP/UNICAMP.

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba
CIBio - Comissão Interna de Biossegurança

CIBioFOP

APROVAÇÃO PARA ATIVIDADES EM CONTENÇÃO COM ORGANISMOS GENETICAMENTE MODIFICADOS E SEUS DERIVADOS

Ofício CIBio-FOP n. A001/2017 Piracicaba, 09 de fevereiro de 2017.

RESPONSÁVEL PELO PROJETO: Prof. Dr. Pedro Luiz Rosalen
com cópia para a chefia do Departamento responsável

Prezado Professor:

O projeto "Papel dos genes *lytTS* e *dltAD* de *Streptococcus mutans* no desenvolvimento e severidade de lesões cariosas em ratos", envolvendo organismos geneticamente modificados do tipo I, sob sua responsabilidade, protocolado sob o número de requerimento R001/2017, foi APROVADO pela Comissão Interna de Biossegurança da Faculdade de Odontologia de Piracicaba – UNICAMP para ser desenvolvido nas dependências do Laboratório da Área de Farmacologia, Anestesiologia e Terapêutica (Nível de Biossegurança NB-1), do Departamento de Ciências Fisiológicas da Faculdade de Odontologia de Piracicaba e da Sala de Animais Geneticamente Modificados (Nível de Biossegurança NB-1), do Biotério da Faculdade de Odontologia de Piracicaba no período de fevereiro de 2017 a fevereiro de 2018. **Aprovação número A001/2017**.

O RESPONSÁVEL PELO PROJETO DEVERÁ:

1) assegurar a plena capacitação da equipe de trabalho e o cumprimento das resoluções normativas (RNs) da CTNBio (disponíveis em www.ctnbio.gov.br). RN 1 [Art. 11 (I, VIII)]

2) manter toda documentação acerca do projeto e da capacitação da equipe de trabalho em arquivo organizado e de pronto acesso, para visitas/inspeções pela CIBio-FOP e/ou órgãos competentes. RN 1 [Art. 11, Art. 18 (IV)]

3) comunicar a CIBio-FOP através de ofício eventuais alterações no projeto e seu local de realização ou na equipe de trabalho. RN 1 [Art.8 (I), Art. 11 (V, IX)]

4) entregar à CIBio-FOP o relatório em formulário padronizado contendo a descrição e o andamento do projeto, impresso em duas vias assinadas/rubricadas pelo responsável pelo projeto e pela chefia do departamento, no primeiro dia útil do mês de fevereiro, anualmente, e quando do encerramento do projeto. RN 1 [Art. 8 (I, IV), Art. 10]

Atenciosamente,

Presidente da CIBio
Prof. Dr. Rafael Nobrega Stipp
Área de Microbiologia e Imunologia - Matrícula: 303502
Departamento de Diagnóstico Oral – FOP/UNICAMP
rafaelns@fop.unicamp.br | (19) 2106-5707

carimbo e rubrica Chefe do Departamento

Prof. Dr. Francisco Carlos Groppo
Chefe do Departamento de Ciências Fisiológicas
Matr. 25.248-4
FOP/UNICAMP

Conteúdo: Aprovação para atividades em contenção com OGM	**Emitente:** Prof. Dr. Rafael Nobrega Stipp
Versão do documento: 15.11	**Aprovado por:** Comissão Interna de Biossegurança FOP/UNICAMP
Número de páginas: 1	**Local:** FOP/UNICAMP (Piracicaba-SP)

PROF. DR. RAFAEL NOBREGA STIPP
Área de Microbiologia e Imunologia
Departamento de Diagnóstico Oral
FOP/UNICAMP

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba
CIBio - Comissão Interna de Biossegurança

Vigência do projeto:
O projeto está autorizado para ser realizado de fevereiro de 2017 a janeiro de 2018.

Outras considerações:
No requerimento não foi informado sobre o armazenamento dos OGMs na FOP/UNICAMP após o transporte. Para realizar o armazenamento, a equipe deverá seguir os procedimentos operacionais autorizados e adotados pela área credenciada, estando em conformidade com a legislação vigente. Ressalto que os experimentos em animais devem possuir a aprovação do órgão competente em ética animal para estudos realizados na FOP/UNICAMP (CEUA/UNICAMP).

Conclusão da análise:
Com base nas informações apresentadas no requerimento nº R001-2017, e tendo em vista que elas atendem aos requisitos necessários para atividades em contenção com OGMs e seus derivados, como sucintamente apresentados nos itens anteriores, sou favorável à aprovação deste requerimento.

Membro da CIBio
Prof. Dr. Antônio Pedro Ricomini Filho

Conteúdo: Parecer técnico	**Modelo de documento:** aprovado pela CIBio-FOP em 03/NOV/2015
Versão do documento: 15.11	**Local:** FOP/UNICAMP (Piracicaba-SP)
Número de páginas: Variável	**Confeccionado por:** Prof. Dr. Rafael Nobrega Stipp

ANEXO D

Aprovação do projeto pelo Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Odontologia de Araraquara – FOAr/UNESP.

FACULDADE DE ODONTOLOGIA DE ARARAQUARA - UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Função de DNA extracelular e de ácidos lipoteicóicos na matriz extracelular de biofilmes cariogênicos.

Pesquisador: MARLISE INÊZ KLEIN

Área Temática:

Versão: 2

CAAE: 31717914.3.0000.5416

Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 748.142

Data da Relatoria: 13/08/2014

Apresentação do Projeto:

Projeto apresentado de forma clara e objetiva, com todos os detalhes necessários para análise.

Objetivo da Pesquisa:

Os objetivos desse projeto são: 1) elucidar a função de eDNA e ALT nas propriedades estruturais e funcionais da MEC (matriz extracelular); 2) determinar a dinâmica de expressão dos genes de S. mutans associados com eDNA e ALT em biofilme cariogênico misto; 3) examinar o papel de eDNA e ALT na colonização dentária e na formação de biofilme in vivo usando um modelo animal.

Avaliação dos Riscos e Benefícios:

Os riscos e benefícios foram avaliados pelo pesquisador responsável.

Comentários e Considerações sobre a Pesquisa:

O estudo proverá base para terapias direcionadas aos elementos importantes na construção da MEC (matriz extracelular) para prevenir e/ou atenuar biofilmes patogênicos.Portanto estudo de relevância para a área em questão.

Considerações sobre os Termos de apresentação obrigatória:

Termos foram apresentados adequadamente.

Endereço: HUMAITA 1680
Bairro: CENTRO **CEP:** 14.801-903
UF: SP **Município:** ARARAQUARA
Telefone: 1633-0164 **Fax:** 1633-0164 **E-mail:** cep@foar.unesp.br; mnagle@foar.unesp.br

FACULDADE DE ODONTOLOGIA DE ARARAQUARA - UNESP

Continuação do Parecer: 748.142

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências para o projeto.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Atendidas pendências de reunião de 26 de junho de 2014, considero APROVADO o protocolo.

ARARAQUARA, 12 de Agosto de 2014

Assinado por:
Maurício Meirelles Nagle
(Coordenador)

Araraquara, 18 de Setembro de 2019

Midian Clara Castillo Pedraza