THAMIRES PRISCILA CAVAZANA SOUZA

Efeito do glicerofosfato de cálcio, associado ou não ao fluoreto, na fisiologia, estrutura, composição da matriz, células cultiváveis, pH e nas concentrações de fluoreto, cálcio e fosfato de biofilmes misto de *S. mutans* e *C. albicans*

Araçatuba

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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Saúde Bucal da Criança.

Orientador: Prof. Titular Alberto Carlos Botazzo Delbem

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Charles Chaplin

Resumo

Souza, TPC. **Efeito do glicerofosfato de cálcio, associado ou não ao fluoreto, na fisiologia, estrutura, composição da matriz, células cultiváveis, pH e nas concentrações de fluoreto, cálcio e fosfato de biofilmes misto de *S. mutans* e *C. albicans***. 2020. 82 f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

RESUMO

O presente estudo teve como objetivo verificar o efeito do glicerofosfato de cálcio (CaGP), associado ou não ao fluoreto (F), na fisiologia, pH e componentes da biomassa e fluido de biofilmes mistos contendo *Streptococcus mutans* e *Candida albicans*, formados *in vitro.* Para formação dos biofilmes colocou-se uma suspenção (1×10^7 células/mL de *C. albicans* + 1×10^8 células/mL de *S. mutans*) em saliva artificial, suplementada com sacarose, em poços de placas de microtitulação. A cada 24 horas, metade do conteúdo da saliva artificial era renovado. Após os períodos de 72, 78 e 96 horas de formação, os biofilmes recebiam tratamentos, por um minuto, com soluções contendo CaGP (0.125, 0.25 ou 0.5%) com ou sem 500 ppm F, e soluções contendo somente F (500 e 1100 ppm F), adotadas como controles positivos, e saliva artificial, considerada como controle negativo. Para estudo da fisiologia microbiológica, após o terceiro tratamento foram realizados os testes de quantificação de células cultiváveis, biomassa total (teste colorimétrico de cristal violeta), atividade metabólica (redução de XTT), análise estrutural do biofilme (microscopia eletrônica de varredura) e quantificação dos componentes da matriz extracelular (proteína, carboidrato e DNA). Todos os ensaios foram realizados em triplicata, em três ocasiões diferentes. Os resultados foram submetidos à análise de variância a um critério, seguida pelo teste Fisher LSD ($p<0.05$). Para estudo de pH e componentes do biofilme, após o período de tratamento, foram analisados F, cálcio (Ca) e fósforo (P) no biofilme total e no fluido do biofilme após a mensuração do pH do biofilme. Em outro conjunto de experimentos, após o terceiro tratamento, o biofilme foi exposto, por 3 minutos, à solução de sacarose a 20%. Esta foi removida e, após 1 minuto, analisou-se o pH do meio e as concentrações de F, Ca, e P tanto na biomassa como no fluido do biofilme. Todos os ensaios foram realizados em triplicata, em três ocasiões diferentes. Os dados foram submetidos à análise de variância a dois critérios, seguida pelo teste de Fisher LSD ($p<0.05$). O CaGP, independente da sua concentração, reduziu significativamente o número de células bacterianas em comparação com o controle negativo. CaGP + F aumentaram a biomassa e a atividade metabólica do biofilme e reduziram os componentes da matriz extracelular avaliada. Os tratamentos com CaGP e 500 ppm F levaram aos maiores valores de pH e concentrações de F e Ca da biomassa do biofilme, antes e após a exposição à sacarose. CaGP, sem F, levou a

maiores concentrações de Ca e P no fluido do biofilme. Assim, é possível concluir que o CaGP interfere na biomassa, metabolismo, composição orgânica e inorgânica, bem como no pH do biofilme testado. Essas informações contribuem para o conhecimento de como CaGP atua na dinâmica de um biofilme relacionado com a doença cárie.

Palavras-chaves: fosfato, flúor, biofilmes, *Streptococcus mutans* e *Candida albicans*

Abstract

Souza, TPC. **Effect of calcium glycerophosphate, associated or not with fluoride, on the physiology, structure, matrix composition, cultivable cells, pH and on the concentrations of fluoride, calcium and phosphate of mixed biofilms of *S. mutans* and *C. albicans*.** 2020. 82 f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

ABSTRACT

The aim of the present study was to evaluate the effect of calcium glycerophosphate (CaGP), associated or not to fluoride (F), on the physiology, pH and components of biofilm biomass and fluid of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*, formed *in vitro*. For the formation of biofilms, a suspension was placed (1×10^7 cells/mL *C. albicans* + 1×10^8 cells/mL *S. mutans*) in artificial saliva, supplemented with sucrose, in wells of microtiter plates. Every 24 hours, half of the artificial saliva content was renewed. At 72, 78 and 96 hours after the beginning of formation, biofilms were treated for one minute with solutions containing CaGP (0.125, 0.25 ou 0.5%) with or without 500 ppm F, and solutions containing only F (500 and 1100 ppm F), adopted as positive controls, and Artificial Saliva, considered as the negative control. For the study of microbiological physiology, after the third treatment, tests were performed: quantification of cultivable cells, total biomass (colorimetric crystal violet test), metabolic activity (XTT reduction), structural analysis of the biofilm (scanning electron microscopy) and quantification of matrix components (protein, carbohydrate and DNA). All assays were performed in triplicate on three different occasions. The results were submitted to one-way analysis of variance, followed by the Fisher LSD's test ($p<0.05$). For the study of pH and biofilm components, after the last treatment, F, calcium (Ca) and phosphorus (P) were analyzed in the total biofilm and in the biofilm fluid after measuring the biofilm pH. In another set of experiments, after the third treatment, the biofilms were exposed for 3 minutes to 20% sucrose solution. This was removed and after 1 minute the biofilms were collected, and the pH of the medium and F, Ca, and P concentrations were determined both in the biomass and in the biofilm fluid. All assays were performed in triplicate on three different occasions. Data were submitted to two-way analysis of variance, followed by Fisher LSD's test ($p<0.05$). CaGP, regardless of their concentration, significantly reduced the number of bacterial cells compared to the negative control. CaGP + F increased the biomass and metabolic activity of the biofilm and reduced the components of the extracellular matrix evaluated. Treatments with CaGP and 500 ppm F led to the highest pH values and F and Ca concentrations of the biofilm biomass, both before and after sucrose exposure. CaGP, without F, led to higher Ca and P concentrations

in the biofilm fluid. Thus, it is possible to conclude that CaGP interferes in the biomass, metabolism, organic and inorganic composition and the pH of the biofilm tested. This information contributes to the knowledge of how CaGP acts in the dynamics of a biofilm related to caries disease.

Keywords: phosphate, fluoride, biofilm, *Streptococcus mutans* and *Candida albicans*.

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LISTA DE ABREVIATURAS E SIGLAS

a.m.	*Ante Meridiem*
Abs	Absorbância
ANOVA	Análise de Variância/*Analysis of Variance*
ATCC	*American Type Culture Collection*
BCA	Ácido Bicinchoninico
BHI	*Brain Heart Infusion*
Ca	Cálcio
$CaCl_2$	Cloreto de cálcio
CaF_2	Fluoreto de cálcio
$CaHPO_4^0$	Fosfato bicálcico
CaGP	Glicerofosfato de cálcio
CFU	Unidades formadoras de colônia/*Colony-forming units*
cm	Centímetros
cm^2	Centímetros quadrados
cm^3	Centímetros cúbicos
CO_2	Dióxido de carbono
CV	Cristal Violeta
DNA	Ácido Desoxirribonucleico
DS	*Degree of saturation*
e.g.	*exempli gratia*
EPS	*Extracellular polymeric substances*
F	Flúor
Fig	Figura
g	Gramas
h	Hora
HA	Hidroxiapatita
HCl	Ácido clorídrico
HF	Ácido fluorídrico
HPO_4^{2-}	Hydrogen phosphate
IA	Atividade Iônica
i.e.	*id est*
KCl	Cloreto de potássio

l	Litro
Log_{10}	Logaritmo de base 10
mg	Miligramas
Mg	Magnésio
min	Minutos
ml	Mililitro
mmol L^{-1}	Millimolar
mV	Milivolt
NaCl	Cloreto de sódio
NaF	Fluoreto de sódio
NaOH	Hidróxido de sódio
NC	Controle Negativo/*Negative Control*
nm	Nanometro
p	*Probability*
P	Fósforo
p.m.	*Post meridiem*
pH	Potencial hidrogeniônico
ppm	partes por milhão
rpm	Rotações por Minuto
s	Segundos
SD	Desvio Padrão/*Standard Deviation*
SDA	*Sabouraud dextrose agar*
SEM	Microscópio eletrônico de varredura/*Scanning electron microscope*
TISAB	*Total Ionic Strength Adjustment Buffer*
UNESP	Universidade Estadual Paulista
UNOESTE	Universidade do Oeste Paulista
XTT	*(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5Carboxanilide)*
µ	Micro
µg	Microgramas
µl	Microlitros
°C	Graus Celsius
g	Gravidade

Sumário

SUMÁRIO

Introdução Geral

INTRODUÇÃO GERAL

A cárie dentária é uma das doenças mais antigas e comuns dos seres humanos. O termo cárie tem origem do latim que significa decadência e foi utilizada para descrever os orifícios nos dentes (RATHEE; SAPRA, 2019). Em resumo, ela é ocasionada pela produção bacteriana de ácidos a partir de carboidratos fermentáveis da dieta, a qual progressivamente desmineraliza a estrutura dentária (CUMMINS; BOWEN, 2006). Atualmente esta doença é considerada um problema de saúde pública, conceituada um processo dinâmico, multifatorial (VELO *et al.*, 2019) que dependente de sacarose e biofilme (SHEIHAM; JAMES, 2015).

O biofilme dental é um conjunto de microrganismos nos quais as células aderem a uma superfície e/ou umas às outras (RATHEE; SAPRA, 2019). A estrutura do biofilme aumenta a cariogenicidade das bactérias produtoras de ácido, protegendo-as da defesa do hospedeiro uma vez que, esse agregado celular é envolto por uma matriz orgânica autoproduzida de polissacarídeos, proteínas e DNA (RATHEE; SAPRA, 2019). Sendo assim, o acúmulo de biofilme faz com que os microrganismos metabolizem eficientemente a sacarose (açúcar ou polímeros, tais como amido) para produzir grande quantidade de ácido láctico capaz de solubilizar o componente mineral do dente e iniciar o processo de cárie (MARSH; MARTIN, 2009; LAMONT *et al.*, 2006).

A bactéria gram positiva *Streptococcus mutans* é um dos principais agentes etiológicos da cárie dentária, devido à sua capacidade de colonizar a superfície dental, metabolizar carboidratos, produzir ácido láctico, além de ter a capacidade de crescer e se multiplicar em ambiente ácido (MARSH; MARTIN, 2009; LAMONT *et al.*, 2006). O processo de formação de biofilme inicia-se com o revestimento da superfície do dente pela película salivar (BOWEN; KOO, 2011; ZIJNGE *et al.*, 2010). Várias adesinas de bactérias odontopatogênicas interagem com as glicoproteínas salivares da película adquirida na superfície dos dentes, por meio de ligação a cátions bivalentes. Na presença de sacarose, as bactérias aderem-se firmemente à superfície como resultado da produção de exopolissacáridos (glucanos) por meio da atividade da enzima glicosiltransferase (MARSH; MARTIN, 2009).

Apesar de *S. mutans* ser citado frequentemente como o principal agente patógeno nos casos de cárie dentária, especialmente em cárie na primeira infância, este microrganismo não age sozinho. A levedura *Candida albicans* é comumente detectado nas superfícies da

mucosa humana e que muitas vezes participa na formação de biofilmes polimicrobianos em superfícies de tecidos moles e acrílicos. Estudo demonstrou que nos casos de cárie precoce da infância, *C. albicans* está frequentemente associada na placa bacteriana patógena (FALSETTA *et al.*, 2014; HAJISHENGALLIS *et al.*, 2017; JEAN *et al.*, 2018; XIAO *et al.*, 2018). Observa-se um aumento de *S. mutans* quando *C. albicans* está presente em biofilmes associados a cárie da primeira infância (KIM *et al.*, 2017).

O meio ácido entre biofilme/dente modifica o equilíbrio mineral entre o esmalte e o ambiente circundante, induzindo a desmineralização, ou seja, a destruição dos cristais de hidroxiapatita (HA) que formam o dente (DAWES, 2003). Ocorrem períodos de desmineralização e remineralização da estrutura dentária e, quando a desmineralização predomina, ocorre a lesão de cárie (ARIFA; EPHRAIM; RAJAMANI, 2019). Como principais componentes dos HA, as concentrações de cálcio e fosfato na saliva e biofilme desempenham um papel fundamental na influência dos processos de desmineralização e remineralização dentária (LI *et al.*, 2014).

A utilização de produtos fluoretados levaram um declínio de cárie no mundo todo (PERES *et al.*, 2019) pois o fluoreto (F) aumenta a resistência do dente ao ataque ácido, sendo um componente essencial na prevenção desta doença (SHAHROOM; MANI; RAMAKRISHNAN, 2019). A utilização dos dentifrícios contendo F atrelado a escovação dos dentes é considerado o melhor método preventivo da cárie dentária, visto que associa a remoção ou desorganização periódica do biofilme dental com as propriedades cariostáticas do F (PESSAN *et al.*, 2006; TENUTA *et al.*, 2009). O F tem seu efeito pela formação de produtos da sua reação com a estrutura dental e formando o fluoreto de cálcio (CaF_2) que, quando depositado no biofilme dental em lesões de cáries iniciais, é capaz de reduzir a sua progressão (CRUZ; ROLLA, 1991). A desvantagem do uso de dentifrícios fluoretados por crianças pequenas é que elas engolem quantidade deste e assim apresentam um risco subsequente de fluorose (WALSH *et al.*, 2019). Este produto pode ser responsável por até 80% da ingestão diária total ideal de flúor, sendo os três primeiros anos de vida mais críticos (MEJÀRE, 2018).

Esta condição impulsiona a busca por estratégias que visam reduzir a quantidade de F e, concomitantemente, potencializa os efeitos preventivos de produtos fluoretados. Destas estratégias, destaca-se a importância do uso de derivados de fosfato de cálcio. Estudos *in vitro* e *in situ* demonstraram que dentifrícios com concentração reduzida de F suplementados com glicerofosfato de cálcio (CaGP) apresentam efeito semelhante à de

um dentifrício convencional (1.100 ppm F) sobre a desmineralização e remineralização do esmalte dental (DO AMARAL *et al.*, 2013; ZAZE *et al.*, 2014a; ZAZE *et al.*, 2014b). Tais achados foram confirmados em um estudo clínico randomizado controlado, no qual a progressão de cárie em dentes decíduos foi semelhante em crianças que utilizaram um dentifrício contendo 500 ppm F e CaGP em comparação a crianças utilizando uma formulação convencional contendo 1100 ppm F (FREIRE *et al.*, 2016). Em relação aos efeitos do CaGP sobre o biofilme dental, estudo *in situ* observou que este fosfato orgânico com 500 ppm F não aumenta a concentração de Ca no fluído do biofilme (NAGATA *et al.*, 2017) e outro observou que não houve diferença entre a composição inorgânica da placa que recebeu CaGP-F e 1100 ppm F (DO AMARAL *et al.*, 2013). Em uma revisão de literatura sobre os efeitos anti-cárie deste polifosfato concluiu que a elevação dos níveis de Ca na placa é a explicação mais provável para o potencial anti-cárie do CaGP (LYNCH, 2004).

Fosfatos inorgânicos como o trimetafosfato de sódio e o hexametafosfato de sódio, mostraram modificações não só em componente orgânicos de biofilmes (CAVAZANA *et al.*, 2019a; HOSIDA *et al.*, 2017) como também em inorgânicos e nos valores de pH antes e após esse biofilme ser exposto à sacarose (CAVAZANA *et al.*, 2019b; SAMPAIO *et al.*, 2018). Com base no exposto, torna-se evidente que o efeito do CaGP-F sobre o esmalte dentário apresenta relatos científicos, enquanto os dados sobre os efeitos da associação CaGP-F sobre o biofilme dental ainda são escassos e conflitantes. Este aspecto reforça a necessidade de estudos adicionais avaliando os efeitos do F e do CaGP sobre o biofilme, especialmente envolvendo métodos analíticos complementares aos utilizados nos estudos supracitados, para uma melhor compreensão dos mecanismos de ação destes íons sobre a cárie dentária.

Desta forma, seria interessante conduzir um estudo *in vitro* avaliando os efeitos da associação entre F e CaGP: sobre a composição orgânica e metabolismo de um biofilme misto de *S. mutans* e *C. albicans*, sobre a retenção de F, P e Ca no biofilme total e no fluido do biofilme (antes e após a exposição deste à sacarose), e sobre o pH deste biofilme. Para abordar o tema proposto, o estudo será apresentado em dois capítulos distintos:

- Capítulo 1: **“Effect of calcium glycerophosphate and fluoride on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*”**
(artigo preparado para a submissão ao periódico Biofouling)

- Capítulo 2: **“Calcium glycerophosphate and fluoride affect the pH and inorganic composition of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*”**
(artigo preparado para a submissão ao periódico The International Journal of Biochemistry & Cell Biology)

Capítulo 1

Effect of calcium glycerophosphate and fluoride on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

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Abstract

This study aimed to evaluate the effect of calcium glycerophosphate (CaGP), associated or not with fluoride (F), on biofilm of *Streptococcus mutans* and *Candida albicans*. Biofilms were treated three times with 0.125, 0.25 and 0.5% CaGP solutions, combined or not with 500 ppm F, in addition to 500 and 1100 ppm F solutions, and artificial saliva. Following, were analyzed physiology and structure of biofilms. CaGP at 0.25 and 0.5% promoted the highest increases in biofilm biomass and metabolic activity, regardless of the presence of F. Fluoride-free CaGP significantly reduced the number of bacteria cells. Also, CaGP and F co-administered reduced the extracellular matrix components evaluated. Concluded that CaGP alone affected the number of *S. mutans* cells and, when associated with F, it promoted changes in the biomass, metabolic activity, and the extracellular matrix components. This information contributes to knowledge of how CaGP acts in the dynamics of caries disease.

Keywords: biofilms, *Streptococcus mutans*, *Candida albicans*, calcium glycerophosphate, fluorides

Introduction

Although dental caries prevalence has declined worldwide, the disease remains as an important public health problem, given its impact on the oral health of the overall population (Pitts et al. 2017). A sugar-biofilm interaction is regarded as the main etiologic factor of dental caries (Sheiham and James 2015), and the bacterium *Streptococcus mutans* consists of the most important species enrolled in the development of this disease (Kanasi et al. 2010). In addition to *S. mutans*, nonetheless, several other microorganisms participate in the development of dental caries, such as *Candida albicans*. This fungus is found in the oral environment and its contribution to the etiology of dental caries has recently been emphasized (Kim et al. 2017; Xiao et al. 2018).

The use of fluoridated products is considered as the main reason for the decline in caries prevalence over the years (Toumba et al. 2019). However, given that approximately 80% of the total daily fluoride (F) intake by toddlers occurs from the ingestion of F-dentifrice, the use of such products has been associated with dental fluorosis development (Walsh et al. 2019; Mejàre 2018).

Aiming to reduce F intake without compromising the protective effect of the ion, studies on the supplementation of F-products with polyphosphate salts have been developed. Calcium glycerophosphate (CaGP), an organic polyphosphate, has shown to markedly enhance enamel de- and re-mineralization (do Amaral et al. 2013; Zaze et al. 2014a, 2014b), but scarce evidence on the effects of CaGP in the dental biofilm is available, mainly focusing on the changes in the ionic composition caused by this polyphosphate (Lynch 2004; Nagata et al. 2017).

Despite recent data have demonstrated the effects of sodium trimetaphosphate associated with F on the composition of biofilms *in vitro* (Cavazana et al. 2019), the effects of CaGP co-administered with F on biofilms remain unknown. Therefore, this study aimed to evaluate the effects of CaGP, associated or not with F, on the physiology and structure of biofilms of dual-biofilms formed by *S. mutans* and *C. albicans*. The study's null hypothesis was that CaGP, associated or not with F, would not affect the biofilm parameters analyzed.

Materials and Methods

Strains and culture conditions

Strains from the American Type Culture Collection (ATCC) of *C. albicans* (ATCC 10231) and *S. mutans* (ATCC 25175) were used in this study. *S. mutans* colonies grown on brain heart infusion agar (BHI Agar; Difco, Le Pont de Claix, France) were suspended in 10 mL of BHI broth (Difco) and incubated (statically) overnight in 5% CO_2 at 37 ºC. For *C. albicans*, colonies previously cultured on Sabouraud dextrose agar (SDA; Difco) were suspended in 10 mL of Sabouraud dextrose broth (Difco) and incubated (aerobically) overnight at 120 rpm and 37 ºC. Both fungal and bacterial cells were centrifuged (6876 × *g*, 5 min) and the cell pellets, washed twice with 10 mL of NaCl (0.85%). The number cells was adjusted in artificial saliva (AS; as described by Lamfon et al. 2003, and modified by Cavazana et al. 2018) at 1×10^7 cells/mL of *C. albicans* using a Neubauer counting chamber, while the number of *S. mutans* was spectrophotometrically (640 nm) adjusted at 1×10^8 cells/mL.

Biofilm formation

Dual-species biofilms were formed in flat-bottom microtiter plates (Costar, Tewksbury, USA), by the addition of the microbial suspensions (1×10^7 cells ml^{-1} for *C. albicans* + 1×10^8 cells ml^{-1} for *S. mutans*) in AS (Cavazana et al. 2019). The plates were incubated in 5% CO_2 at 37 ºC for 96h. Every 24 h, half of de well content was renewed with AS.

Treatment of the biofilms

A previous study showed that 0.25% CaGP combined with 500 ppm F promoted results similar to 1100 ppm F in relation to enamel demineralization (Zaze et al. 2014a). Therefore, to evaluate dose-response effects in the biofilm, CaGP was used at the concentration above, as well as half and double of this, resulting in the following experimental groups: CaGP (Sigma-Aldrich, St Louis, MO, USA) at 0.125, 0.25 and 0.5%, associated or not with 500 ppm F. In addition, solutions containing 500 and 1100 ppm F were also evaluated, while pure AS was tested as negative control (NC).

The biofilms were treated after 72, 78 and 96 h of their formation, resulting in three treatments (Cavazana et al. 2019). For this, all AS was removed and the biofilms

were exposed to the experimental solutions for 1 min (Koo et al., 2005). Next, the solutions were removed from the wells and the biofilms received a new AS (Cavazana et al. 2019). After the last treatment (96 h after the beginning of formation), the biofilms were gently washed with 0.85% NaCl, and the quantitative and qualitative experiments were performed. All tests were performed in triplicate and repeated at three independent moments (Cavazana et al. 2019).

Biofilm biomass evaluation

Biofilm biomass was evaluated by the crystal violet (CV) staining assay, as described by Monteiro et al. (2011). For this, the biofilms were fixed with 99% methanol (Sigma-Aldrich) for 15 min, stained for 5 min with 1% CV (Sigma-Aldrich), and de-stained through exposure to 33% acetic acid (Sigma-Aldrich). Absorbance values were read (570 nm) and represented as a function of the area of the wells (absorbance/cm^2). AS without microbial cells was used as blank.

Metabolic activity evaluation

The effect of the treatment solutions on the metabolic activity of the biofilms was performed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)- 5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma-Aldrich) reduction method (Fernandes et al. 2016). Briefly, XTT and phenazine methosulphate (Sigma-Aldrich) solutions were combined and pipetted into the wells, and the plates were then incubated at 37 ºC (for 3h at 120 rpm), protected from light. The absorbance values were measured at 490 nm (absorbance/cm^2). AS without microbial cells was used as blank.

Cultivable cells evaluation

Colony-forming units (CFUs) counting was used to evaluate the number of cultivable cells. After the last treatment, the biofilms were resuspended in NaCl (0.85%) and scraped from the wells. Biofilm suspensions were then serially diluted in 0.85% NaCl and plated on CHROMagar Candida (Difco), for *C. albicans* counting, and on BHI agar supplemented with amphotericin B (7 µg/mL; Sigma-Aldrich), for *S. mutans* counting. Agar plates were incubated for 24–48h at 37 ºC, and the number of CFUs was expressed as $\log_{10}$ CFU/cm^2 (Fernandes et al. 2016).

Evaluation of extracellular matrix composition

For this assay, dual-species biofilms were grown in s6-well plates (Costar) containing 4 mL of the microbial suspension, as previously detailed. After the last treatment, the biofilms were resuspended in 0.85% NaCl, scraped from the wells, and the liquid phase of the extracellular matrix was extracted by sonication (for 30 s at 30W) (Silva et al. 2009). Protein determination of the extracellular matrix was performed by the bicinchoninic acid method (Kit BCA; Sigma-Aldrich), using bovine serum albumin as the standard (Silva et al. 2009). The carbohydrate content was quantified as described by Dubois et al. (1956), with glucose as the standard. For the evaluation of the nucleic acids content, 1.5 mL of the liquid phase of the extracellular matrix was spectrophotometrically analyzed (at 260 and 280 nm) in a Nanodrop Spectrophotometer (EONC Spectrophotometer of EONC, Biotek, Winooski, VT, USA) (Monteiro et al. 2013). Both protein, carbohydrate and nucleic acids values were expressed as mg/g dry weight of biofilm.

Structural analysis of the biofilm

Scanning electron microscopy (SEM) was performed to evaluate the effect of the treatment solutions on the structure of the biofilms. In brief, dual-species biofilms of *C. albicans* and *S. mutans* were formed in 24-well plates and exposed to the experimental solutions, as previously described. After the last treatment, the wells were gently washed with NaCl (0.85%), and the biofilms were dehydrated using the following series of ethanol concentrations: 70% for 10 min, 95% for 10 min, and 100% for 20 min, followed by air drying for 20 min (Silva et al. 2013). Next, the bottom of each well was cut with a flame-sterilised scalpel blade (number 11, Solidor, Lamedid Commercial and Services Ltd, Barueri, Brazil), and the biofilms were coated with gold and evaluated using an electron microscope (S-360 microscope, Leo, Cambridge, MA, USA).

Statistical analysis

All assays was performed in triplicate and repeated at three different moments. Data passed normality and homogeneity tests (Shapiro-Wilk), and were submitted to ANOVA, followed by Fisher's LSD *post hoc* test. The statistical analysis was performed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA), adopting $p<0.05$ as statistically significant.

Results

Biofilm biomass evaluation

Treatment with 500 ppm F alone and CaGP at 0.25 and 0.5% (associated or not with F) led to increases in the biofilm biomass in comparison to NC (p<0,001; Figure 1a). The highest biomass values were observed for biofilms treated with CaGP at 0.5% (1.20 abs/cm^2) and CaGP at 0.5% associated with F (1.14 abs/cm^2). Biofilms treated with 1100 ppm F, as well as those exposed to 0.125% CaGP (with or without F) did not differ from the NC group (p>0,05; Figure 1a).

Metabolic activity evaluation

All treatments promoted significant increases in the metabolic activity of the biofilms in comparison to NC, and the solutions containing only F (500 ppm F and 1100 ppm F) did not differ from each other (p>0,05; Figure 1b). Regarding the groups treated with CaGP-containing solutions, a dose-response effect was observed, given that the higher the concentration of CaGP, the higher the metabolic activity of the biofilms. Groups treated with CaGP associated with F showed higher values of metabolism in comparison to their counterparts without F [Figure 1b].

Cultivable cells evaluation

All CaGP-containing solutions associated with F did not lead to significant reductions in the CFUs of *S. mutans* compared to NC (p>0.05; Figure 1c), while treatments with CaGP alone significantly decreased the CFUs in comparison to NC (p<0.001; Figure 1c). All CaGP solutions without F led to higher reductions in the CFUs of *S. mutans* compared to the groups exposed to 500 ppm F, while the highest decrease was observed for the 1100 ppm F group. Regarding *C. albicans*, the exposure to all treatment solutions did not significantly affect the number of CFUs [Figure 1d].

Evaluation of extracellular matrix composition

Regarding the amount of proteins, the lower values were observed for the groups treated with CaGP at 0.25% and 0.5% + F, with no significant difference in comparison to 1100 ppm F group (p>0,05; Table 1). No significant differences were observed for

the biofilms treated with CaGP at 0.125% and 0.25% with F, in comparison to 500 ppm F (p>0,05; Table 1). As for carbohydrates, CaGP at 0.5% + F promoted the highest reductions (~23% than the NC, and ~42% than the 1100 ppm F group). For nucleic acids content, biofilms exposed to 1100 ppm F presented lower concentration in comparison to 500 ppm F group. CaGP at higher concentrations led to higher reductions in relation to the NC, while lower values of nucleic acids were found in groups treated with CaGP associated with F [Table 1].

Structural analysis of biofilms

In general, structural differences were not noted among biofilms treated with the different solutions, and all biofilms exhibited coccus attached to yeasts and hyphae, forming dense networks [Figure 2]. The image of the biofilm treated with 1100 ppm F suggests lower number of bacterial cells than the other groups [Figure 2].

Discussion

F is an important agent for the control of dental caries (Frencken et al., 2017), but excessive ingestion can lead to dental fluorosis, reason by which strategies to reduce systemic exposure without compromising its protective effect have been studied. One possible alternative is combining lower F concentrations with CaGP, which is known to promote a similar protective effect in comparison to a conventional dentifrice (1100 ppm F) on enamel de- and re-mineralization (do Amaral et al. 2013; Zaze et al. 2014a, 2014b). In the present study, CaGP, associated or not with F, influenced some of the biofilm parameters analyzed, thus leading to the partial rejection of the study's null hypothesis.

According to Lynch (2004), exposure to CaGP leads to higher levels of calcium (Ca) and phosphate (P) in the biofilm, which is the most likely explanation for the anti-caries potential of this polyphosphate. The ability of the microorganisms within biofilms to detect changes in components of the medium is an important task that allows pathogens to adjust efficiently to the environment, thus triggering the expression of genes responsible for virulence and resistance (Islam 2020). Tolerance to Ca can play a crucial role in cariogenic bacteria (Astasov-Frauenhoffer et al. 2017), since this ion alters the cellular metabolism of microorganisms and their ability to detect and respond to

environmental concentration, a crucial aspect of bacterial pathogenesis (Rosch et al. 2008).

Bacterial influx systems for cations contribute to the pathogenesis and efflux systems have been characterized mainly in contaminated environmental sites. High concentrations of calcium in the medium are toxic to gram-positive bacteria, and their survival depends on the Ca^{2+} exporter (Rosch et al. 2008). Due to the influx of Ca channels, there may be an increase in this cation in the cell cytosol (Ripoll et al. 2004) and for efflux to occur, the pumps require ATP to maintain the appropriate intracellular concentration (Dominguez 2004). The consequences of high Ca^{2+} growth characterized the activation of autolysis and the induction of competence (Trombe et al. 1992). The increase in Ca concentrations in the medium triggers a change in the bacterium's DNA and cell lysis. This modification in the genetic material is related to the competence to inhibit the uptake of Ca. Bacteria that are able to capture the exogenous genetic material with the competence can overcome lysis and survive in environments with high concentration of Ca (Trombe et al. 1992). These changes related to Ca and bacteria can be seen in the results found. Biofilms treated only with CaGP showed a decrease in the number of *S. mutans* and higher DNA values in the medium, when compared to the same CaGP concentrations associated with 500 ppm F (Figure 1c and Table 1). Thus, it is suggested that the treatments led to higher concentrations of Ca that induced cellular changes resulting in cell lysis and release of genetic material that may have competence for survival in a high Ca environment.

The response of *C. albicans* to the increases in Ca concentrations may be reflected in the increased metabolic activity of the biofilm that occurred when the treatment contained only CaGP, which showed a decrease in *S. mutans* cells (Figure 1b and 1c). In this microorganism, Ca^{2+} channels open up and allow Ca^{2+} to passively enter the cytosol due to the concentration gradient, from the extracellular space or intracellular stores. The Ca^{2+}/H^{+} antiporting devices use the driving force of the proton, and Ca^{2+} pumps use ATP to move Ca^{2+} against a concentration gradient outside the cytosol in order to maintain the ideal internal levels. *C. albicans* also has Ca-ATPase in the vacuolar membrane to overcome the toxic effect of cations, storing its excess in its vacuoles (Yu et al. 2014) and this is essential for its growth in media with a high Ca^{2+} content (Lange et al. 2020). The excess of external Ca^{2+} affects the formation of hyphae, resulting in the formation of pseudo-hyphae and thus decrease the virulence of this fungus (Sakaguchi et al. 1997).

F alone also increased the metabolic activity of the biofilms, as previously reported (Nassar and Gregory 2017). The groups that received CaGP associated with F presented the highest metabolism, what can be explained due to the cumulative effect of Ca (from CaGP) and F on such microorganisms. CaGP associated with F did not interfere in the number of *S. mutans* cells in comparison to NC [Figure 1c]. This might be related to CaF_2 formation (resulted from Ca and F availability), thus reducing the availability of Ca in the medium. In addition, this interaction also interfered on the amount of F in the environment, which annulled the effect found in the group of 500 ppm F without polyphosphate, supporting this hypothesis. Regarding *C. albicans*, no difference on the CFUs number was observed, possibly because this fungus was not affected by the amount of ions provided from CaGP and CaGP+F treatments [Figure 1d].

Treatments with a high Ca content lead to an increase of calcium bound to the surface of the dental biofilm bacteria (Leitão et al. 2018). Calcium shows interactions with surface proteins and forms ionic bridges between negatively charged macromolecules, improving cell aggregation and strengthening biofilm matrices. It is suggested that the proximity of neighboring cells depends on the calcium bridge (Rose 2000) and presence of Ca leads to a connection between molecules (Korstgens et al. 2001). This change in biofilm may justify the increase in biomass found for biofilms treated with CaGP by 0.25 and 0.5%, regardless of the presence of F, compared to NC [Figure 1b]. It is suggested that these binding of Ca and microorganisms are able to retain new compounds, thus leading to an increase in biofilm biomass. This hypothesis is in line with the quantitative results of the present study, considering that the increases in biomass was not accompanied by increases in the number of cells or in the components of the extracellular matrix. In addition, a structure with less spaces between cells, observed in the SEM analysis for these groups possibly results from calcium bridges within the biofilm [Figure 2].

The structural rigidity for the protection from the external environment, and the control of gene regulation and nutrient adsorption are important functions of the extracellular matrix (Hobley et al. 2015). Sucrose consists of an essential component for the production of extracellular matrix, thus affecting biofilm growth (Tomé et al. 2017), and for this reason it was added to saliva in the present study. The treatments carried out with CaGP and F were effective in reducing carbohydrates, proteins and nucleic acids from the extracellular matrix. Similar results were found in a previous study that tested the effect of aninorganic polyphosphate on dual-species biofilm of *S. mutans* and *C.*

albicans (Cavazana et al. 2019). An *in situ* study also showed a significant reduction in the concentrations of extracellular polymeric substances (EPS) in biofilms exposed to CaGP and F (do Amaral et al. 2013), thus reinforcing the present findings.

The presence of water-insoluble glucans in the biofilm matrix is a relevant factor in the biofilm's pathogenicity related to dental caries (Koo et al. 2013). These polysaccharides hinder the penetration of drugs and produce acidic areas, favoring the demineralization of the dental surfaces (Xiao et al. 2012). In this sense, the reduction in carbohydrates consists of a favorable aspect for dental caries control. Lower protein and nucleic acids values also play an important role in dental caries, given their involvement on the fixation of the biofilm on the surfaces, allowing connection between cells, horizontal transfer of genes between cells and other functions of the extracellular matrix (Flemming and Wingender 2010).

It is important to emphasize, nonetheless, that a dual-species biofilm model does not fully represent a multispecies biofilm found in the oral environment. Despite the limitation of this biofilm model, the results obtained provided additional data on the mechanisms by which CaGP interferes in the dynamics of dental caries, stimulating further studies with this polyphosphate.

Conclusions

CaGP alone reduced the number of CFUs of *S. mutans*, but when associated with F it was not able to promote significant reductions in the number of bacterial cells. This polyphosphate did not interfere in the number of fungal cells of the tested biofilm. In addition, CaGP was able to increase both the metabolic activity and the biomass of the dual-species biofilm. Regarding the extracellular matrix, when the CaGP was associated with F, it reduced all the components analyzed. Given the above, it is concluded that CaGP, associated or not with F, does not change only the dental structure, as reported in the literature, but is able to modify the physiology of the biofilm related to this disease. Thus, the use of this polyphosphate could be promising, since it modifies the behavior and composition of an essential component for the development of caries, the biofilm.

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Table 1. Mean values (SD) of each component of the extracellular matrix of dual-species biofilms obtained after treatment with different concentrations of CaGP, associated or not to F

Matrix component (mg/g of biofilm dry weight)	Groups								
	NC	**500 F**	**1100 F**	**0.125 CaGP**	**0.25 CaGP**	**0.5 CaGP**	**0.125 CaGP+F**	**0.25 CaGP+F**	**0.5 CaGP+F**
Proteins	34.39^{A} (3.87)	29.29^{B} (2.47)	26.51^{CD} (2.00)	27.64^{BC} (2.19)	24.17^{D} (2.61)	30.38^{B} (2.42)	27.49^{BC} (3.48)	30.16^{B} (3.61)	24.47^{D} (2.45)
Carbohydrates	$250,20^{A}$ (27,44)	132.81^{BC} (10.98)	143.16^{B} (19.83)	165.65^{E} (16.12)	190.47^{F} (7.45)	161.75^{E} (10.01)	116.25^{D} (4.19)	122.92^{CD} (9.23)	59.87^{G} (3.02)
DNA	18.19^{A} (1.15)	16.44^{B} (0.97)	13.82^{C} (1.19)	18.20^{A} (1.02)	17.20^{AB} (1.64)	13.36^{C} (1.36)	13.65^{C} (1.02)	12.31^{D} (1.33)	10.63^{E} (1.03)

Different upper case letters symbolize statistical differences among the groups ($p<0.05$).

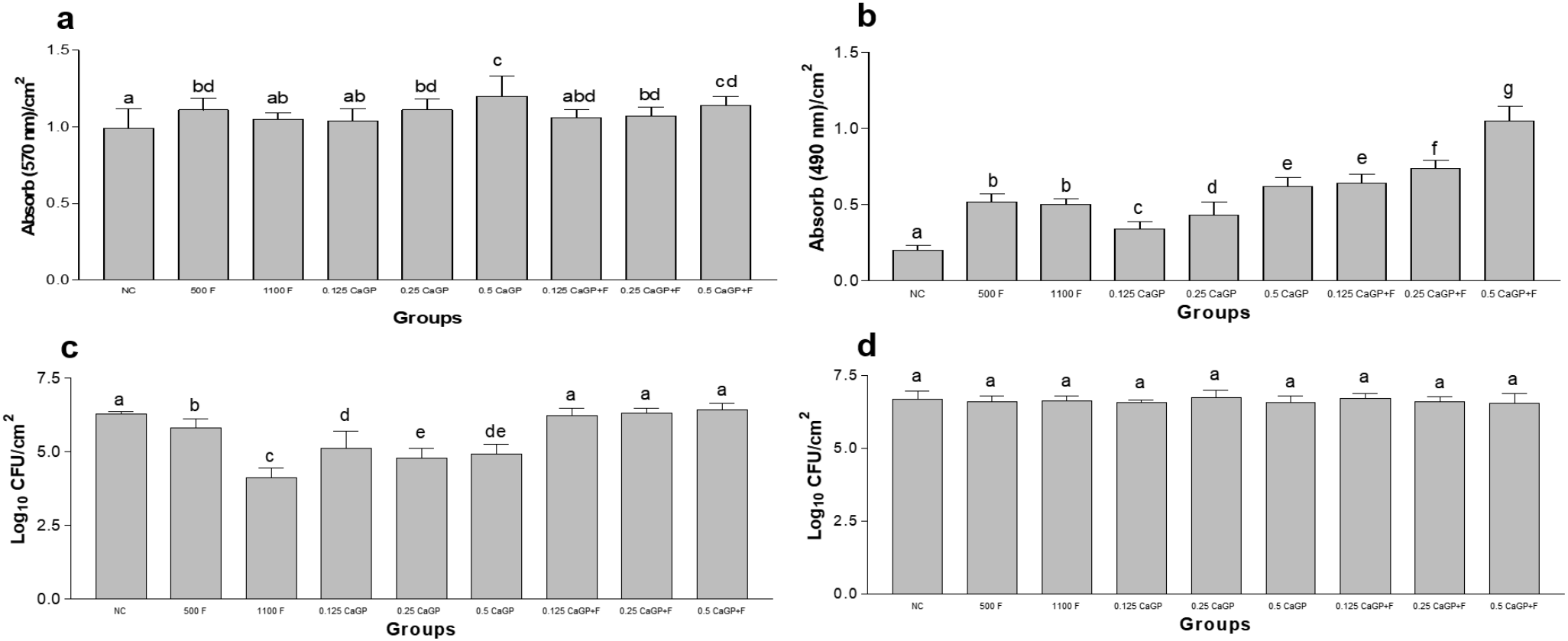

Figure 1. Absorbance values per cm^2 obtained for quantification of total biomass (a) and metabolic activity (b), and logarithm of colony-forming units per cm^2 for *S. mutans* (c) and *C. albicans* (d) in dual-species biofilms. NC: negative control (untreated biofilms). Error bars denote the standard deviations of the means. Different letters symbolize statistical differences among the groups ($p<0.05$).

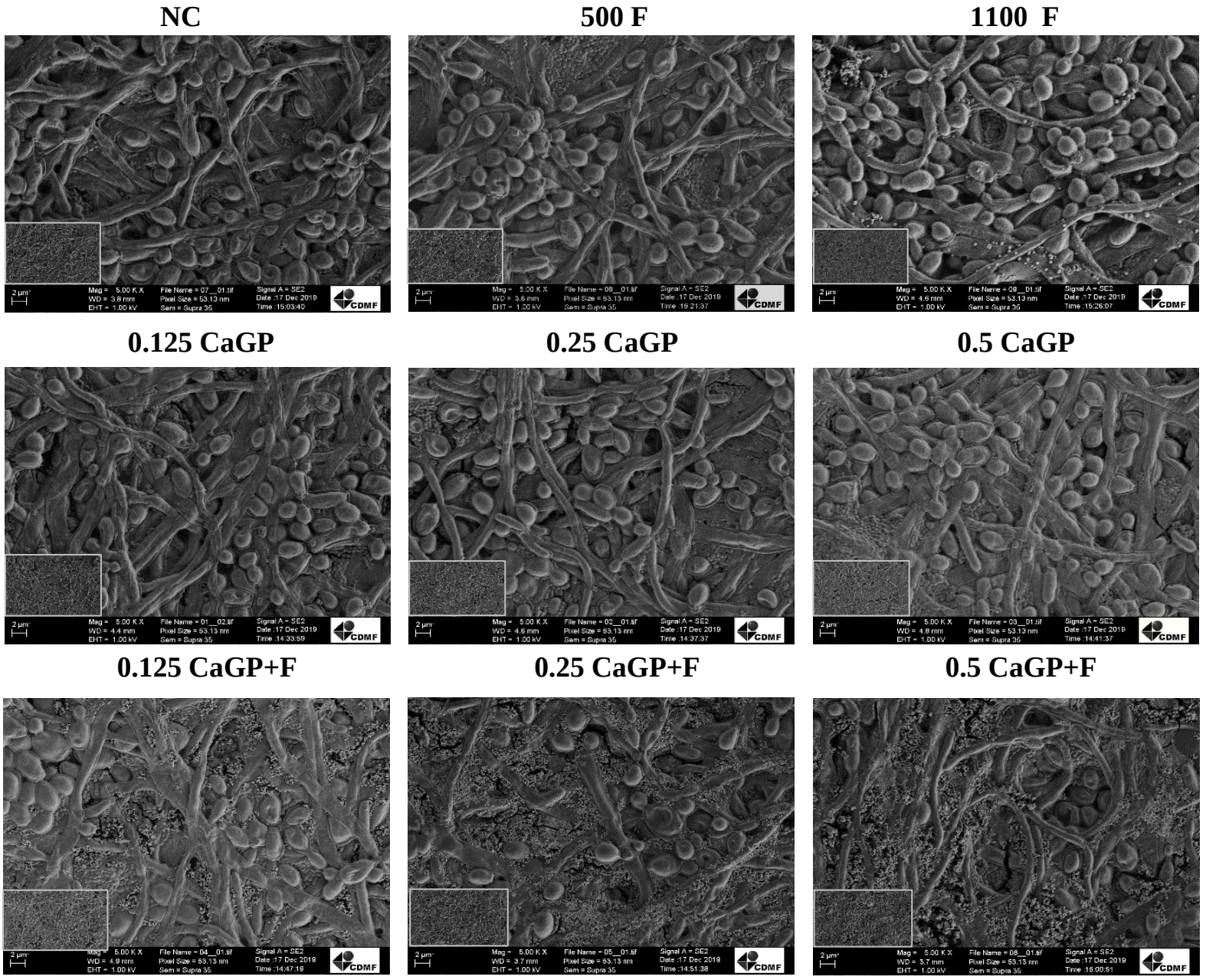

Figure 2. SEM images of dual-species biofilms of *C. albicans* and *S. mutans* after treatment with different experimental solutions. Magnification: 5,000 ×. Bars: 2mm.

Capítulo 2

Calcium glycerophosphate and fluoride affect the pH and inorganic composition of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

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Abstract

This study evaluated the influence of calcium glycerophosphate (CaGP), associated or not with fluoride (F), on the pH and on F, calcium (Ca) and phosphorous (P) concentrations of mixed biofilms of *S. mutans* and *C. albicans*, before and after exposure to sucrose. The biofilms received three treatments (72, 78 and 96 hours after the start of their formation), at three CaGP concentrations (0.125, 0.25 or 0.5%), with or without F at 500 ppm. Solutions containing 500 and 1100 ppm F, and artificial saliva were also tested as controls. Biofilm pH was measured, and the concentrations of F, Ca, P and CaGP were determined (solid and fluid phases). In a parallel experiment, after the third treatment, treated biofilms were exposed to a sucrose solution, and the pH of the medium, F, Ca, P and CaGP were determined. Data were submitted to two-way ANOVA, followed by Fisher LSD's test ($p<0.05$). Treatments with CaGP and 500 ppm F led to the highest pH values and F and Ca concentrations in the biofilm biomass, both before and after sucrose exposure. CaGP without F led to higher Ca and P concentrations in the biofilm fluid. It was concluded that CaGP increases F, Ca and P in the biofilm, and its presence promotes an increase in the pH of the medium, even after exposure to sucrose. These data contribute to elucidate how CaGP acts on the dental caries dynamic.

Keywords: biofilms, *Streptococcus mutans*, *Candida albicans*, calcium glycerophosphate, Fluorides, Phosphate

1. Introduction

Dental caries consists of a sugar-biofilm dependent disease (Sheiham and James, 2015), and remains one of the main public health problem in several populations worldwide (Asokan et al., 2019). Several microorganisms are enrolled in dental caries onset and progression, especially the bacteria *Streptococcus mutans* (Kanasi et al., 2010). Nonetheless, recent studies have stated the role of other microorganisms, including the fungus *Candida albicans*, on caries dynamic (Pereira et al., 2017; Xiao et al., 2018).

Among several strategies for caries prevention, fluoride (F) is an essential agent, given that it increases dental resistance against acid challenges (Shahroom et al., 2019), reason by which preventive strategies include F therapy in a variety of modalities, both for professional- and self-administration. Other ions also take part in caries dynamics, including phosphorus (P) and calcium (Ca), which are known to exert a relevant role on dental de- and -remineralization process when present in the biofilm and biofilm fluid (ten Cate and Buzalaf, 2019).

Despite the unquestionable benefits of F in caries control, excessive intake of this ion may lead to dental fluorosis development. In order to reduce F ingestion from dentifrices, measures including the reduction of the amount of the product applied on the brush (Walsh et al., 2019) or reducing F concentration of the product have been suggested. Given that evidences to attest such recommendations remain scarce (Sampaio et al., 2020), other strategies such as the supplementation of dentifrices with polyphosphate salts have been intensively studied over the last decade.

Calcium glycerophosphate (CaGP) is an organic phosphate salt that potentiates the effects of F on enamel de- and re-mineralization (do Amaral et al., 2013; Zaze et al., 2014ab), allowing the reduction of the F content in the formulation without compromising its clinical efficacy compared to a conventional (1100 ppm F) formulation (Freire *et al.*, 2016). Data from an *in situ* study demonstrated that the inorganic composition of biofilms exposed to a low-fluoride dentifrice (500 ppm F) associated to CaGP did not significantly differ from that treated with a conventional dentifrice (1100 ppm F) (do Amaral et al., 2013), Surprisingly, however, a more recent *in situ* study from the same group demonstrated that treatment with dentifrice containing CaGP and 500 ppm F did not increase Ca concentration in the biofilm fluid (Nagata et al., 2017).

Considering the inconsistencies above, and given the need for further information regarding the mechanism by which CaGP affects biofilms, this study aimed to evaluate

in vitro the effect of CaGP, associated or not with F, on the inorganic composition (Ca, P and F) and pH of dual-species biofilms of *S. mutans* and *C. albicans*, prior and after the exposure to sucrose, to mimic a cariogenic challenge. The study's null hypothesis was that CaGP, associated or not with F, would not affect the biofilms on the parameters evaluated.

2. Materials and Methods

2.1. Strains and biofilm formation

C. albicans (10231) and *S. mutans* (25175) strains from the American Type Culture Collection (ATCC) were used in this study. For *S. mutans*, colonies grown on brain heart infusion agar (BHI Agar; Difco, Le Pont de Claix, France) were suspended in 10 mL of BHI broth (Difco), and statically incubated overnight in 5% CO_2 (37 ºC). For *C. albicans*, colonies previously cultured on Sabouraud dextrose agar (SDA; Difco) were suspended in 10 mL of Sabouraud dextrose broth (Difco) and aerobically incubated overnight at 120 rpm (37 ºC). Fungal and bacterial cells were centrifuged (8000 rpm) for 5 min, and the cell pellets washed twice with 10 mL of saline solution (NaCl 0.85%). The number of cells was adjusted in artificial saliva (AS), as described by Lamfon et al. (2003), and modified by Cavazana et al. (2018), with the following components: sucrose (Sigma-Aldrich, St Louis, MO, USA), yeast extract (Sigma-Aldrich), bacteriological peptone (Sigma-Aldrich), mucin type III (Sigma-Aldrich), NaCl (Sigma- Aldrich), $CaCl_2$ (Sigma-Aldrich), and KCl (Sigma-Aldrich), pH 6.8. *C. albicans* cells were adjusted at 1×10^7 cells/mL, using a Neubauer counting chamber, while the number of *S. mutans* was spectrophotometrically (640 nm) adjusted at 1×10^8 cells/mL. Then, the dual-species biofilms were grown in 6-well plates at 5% CO_2 (37 ºC), and the medium was renewed every 24 h by removing half of the well content and adding an equal volume of fresh AS (Cavazana et al., 2019a; Cavazana et al., 2019b).

2.2. Treatment solutions and pH measurement

Solutions containing CaGP and/or F were prepared at different concentrations according to a previous study (Zaze et al., 2014a), resulting in the following experimental groups (solutions): CaGP (Sigma-Aldrich) at 0.125, 0.25 and 0.5%, associated or not with 500 ppm F, 500 and 1100 ppm F (without CaGP), and AS (negative control).

The biofilms were treated based on a protocol proposed by Koo et al. (2005). For this, 72, 78 and 96 h after the beginning of their formation, the biofilms were treated with 2 mL of the treatment solutions, during 1 min, totaling three treatments. After the last treatment, the biofilms were gently washed with 1 mL of AS (10 seconds). Following, biofilms were scraped with a cell scraper (Kasvi) and transferred to microtubes (MCT-200-C-Axygen). The pH measurement was performed with a pH electrode (PHR-146 Micro Combination pH Electrode - Fisher Scientific), previously calibrated with standards (pH 4 and 7) (Cavazana et al., 2018; 2019b).

2.3. Sucrose exposure

For biofilms exposed to sucrose, after the last treatment, a 20% sucrose solution were gently placed in the wells, and removed after 1 min, simulating a cariogenic challenge (Cavazana et al., 2018). Then, the pH measurement was performed, as previously reported.

2.4. Evaluation of the inorganic components of the biofilm fluid

The microtubes containing the scraped biofilms were centrifuged (15267 × *g*) at for 5 min (4º C), and the biofilm fluid was collected for Ca, F and P analyzes (Cavazana et al., 2019b).

F content was measured using an ion-selective (Orion 9409 BN) and a reference electrode (Orion 900100), both coupled to a potentiometer (Orion – Thermo Scientific), after buffering with TISAB II, at a 1:1 ratio. The electrodes were calibrated with solutions standards with 0.09, 0.18, 0.36, 0.72, and 1.44 μg F/mL (for biofilms treated with F-free solutions) and 6.25, 12.5, 25, 50 and 100 μg F/mL (for biofilms treated with F-containing solutions).

Ca concentrations were spectrophotometry evaluated on a plate reader (EONC Spectrophotometer of EONC, Biotek, USA) at wave length of 650 nm. For this, 5 μL of the standards or samples, 50 μL of deionized water and 50 μL of Arsenazo III were used (Vogel et al., 1983).

Total phosphorus was measured as described by Fiske and Subbarow (1925). The determination of P from CaGP was performed using the protocol proposed by Anderson et al. (1977). For the samples that were exposed to sucrose, the boiling-water

bath process for the evaluation of P from CaGP was replaced by maintaining the solutions at 60 °C for 6 h (Cavazana et al., 2019b).

2.5. Calculation of ionic activities and degree of saturation from the biofilm fluid

The ionic activities (IA) of species involved in enamel remineralization ($CaHPO_4^0$, HPO_4^{2-}, and HF^0) were determined from calcium, fluoride, and phosphorus concentrations (mmol/L) in the biofilm fluid of each group (Cochrane et al., 2008). In addition, the degree of saturation (DS) of the solid phases of hydroxyapatite (HA) and calcium fluoride (CaF_2) was determined. All calculations were performed for conditions at 37°C, pH and density of 1.0g/cm^3 using the PHREEQC Interactive software (version 2.18.3, U.S. Geological Survey Branch of Information Services, Denver, CO, USA).

2.6. Evaluation of the inorganic components of the biofilm biomass

F, Ca and P from the biofilm biomass were determined according to the above-mentioned for biofilm fluid measurements. The extraction of the inorganic components from the biomass was performed by adding 0.5 mol/L HCl to the microtubes containing the biofilms at the proportion of 0.5 mL/10.0 mg plaque wet weight (Cury et al., 2000), and homogenizing. The resulting mixture was submitted to a 3 hours constant stirring (120 rpm), at room temperature and then centrifuged (11.000 × *g*) for 1 minute (Nobre Dos Santos et al., 2002). A known amount of the liquid was removed and the same volume of 0.5 mol/L NaOH was added.

2.7. Statistical analysis

Data were analyzed using SigmaPlot (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). Shapiro-Wilk's test was used to verify the normality of the data. Two-way analysis of variance, followed by Fisher LSD's tests was performed, adopting a significance level of 5%.

3. Results

3.1. Biofilm pH

After exposure to sucrose, a reduction in the pH values of the biofilms was observed for all biofilms, except for those treated with phosphate alone (Table 1). The lowest pH values were observed for the NC (p>0.05) and the highest, for the 0.5 CaGP/F group, both

before and after exposure to sucrose. A direct dose-response relationship was observed between F concentration in the treatment solutions (NC, 500 ppm F and 1100 ppm F) and the resulting biofilm pH (Table 1).

3.2. Biofilm fluid

After exposure to sucrose, all components analyzed significantly decreased, for all treatment solutions ($p<0.001$; Figure 1). Regarding F, the higher the concentration of CaGP, the lower the amount of F found in the fluid for groups treated with CaGP and F in association. In addition, these groups showed F values lower than the 500 ppm F group (without CaGP), both before and after sucrose (Figure 1). Regarding Ca concentrations, the highest values were found in groups treated with CaGP only, and with a dose-response in relation to CaGP concentration (Figure 1). Also, the highest amounts of P in the fluid were also observed in the groups that received CaGP without F, but only prior to sucrose, and were not influenced by CaGP concentrations (Figure 1). Furthermore, CaGP levels in the fluid reflected those in the treatment solutions. The F concentrations in the treatment solutions have also been shown to influence F and Ca found in the fluid; the higher the concentration of F in the treatment solutions, the higher the F values and the lower Ca values in the fluid (Figure 1).

3.4. Degree of saturation of biofilm fluid and calculation of ionic activities

The degree of saturation of the biofilm in relation to HA decreased after exposure to sucrose in all groups (Table 2). This reduction also occurred in relation to CaF_2 saturation, except for the 0.25% CaGP group, which did not present a significant difference in the values found between before and after sucrose ($p=0.728$) (Table 2). The higher the phosphate concentration, the greater the saturation of the biofilm in relation to HA, and the association with F led to the highest values (Table 2). Regarding the F concentration, 1100 ppm had the lowest saturation values for both HA and CaF_2, compared to the 500 ppm F group before sucrose.

The possibility of $CaHPO_4^0$ formation was higher in the groups that presented CaGP ($p<0.001$) in the treatment before the exposure of this biofilm to sucrose. After exposure, there was a reduction in the possibility of forming this component and treatments with CaGP 0.5 showed the highest values (Table 3). The lower concentrations of CaGP, associated with F, stimulated higher values of HPO_4^{2-} before the exposure of the biofilm to sucrose (Table 3). The presence of CaGP did not modify the possible formation of HF

in relation to the control ($p > 0.05$) and, the highest values of this were found in treatments with F alone, both before and after sucrose (Table 3).

3.5. Biofilm biomass

Regarding the biofilm biomass, exposure to sucrose led to a decrease in P for all groups and for Ca in groups NC, 500 and 1100 ppm F ($p < 0.001$; Figure 2). After exposure to sucrose, F and Ca concentrations in the biofilm of the 0.5 CaGP/F group remained unaltered, but increased for the 0.125 and 0.25 CaGP/F groups (Figure 2). The amount of F and Ca in the biomass before exposure to sucrose showed a direct dose-response relationship with the concentration of CaGP in treatments containing CaGP+F. As for P (Figure 2), the concentration of CaGP directly interfered in P concentrations in biomass when not associated with F, prior to sucrose exposure. As for CaGP levels in the biomass, the CaGP/F groups promoted a higher amount of CaGP compared to groups exposed to CaGP only, and being proportional to those observed in the treatment solutions before sucrose (Figure 2).

4. Discussion

The combination of CaGP and F has been shown to enhance the protective and remineralizing effects of toothpastes under controlled conditions (do Amaral et al., 2013; Zaze et al., 2014a, 2014b), allowing the use of lower F concentrations without compromising the clinical efficacy of the product in the primary dentition (Freire et al., 2016). Nonetheless, the conflicting evidence on the effects CaGP and F when co-administered on biofilms motivated the present study, which demonstrated that CaGP increased biofilm pH both before and after sucrose and, when associated with F, it influenced the inorganic composition of the biofilm (biomass and biofilm fluid), thus leading to the rejection of the study's null hypothesis.

Dental caries is the result of the demineralization of the mineral crystal of the dental structure by organic acids produced by bacteria after the metabolization of fermentable carbohydrates, mainly sucrose (Pitts et al., 2017). Biofilm pH is closely linked to demineralization, given that increases in acid concentrations in the fluid phase of the biofilm make it unsaturated in relation to the tooth mineral, thus resulting in mineral loss from the dental structure (Pitts et al., 2017). In the present study, solutions containing CaGP + 500 ppm F were able to maintain higher pH values compared to the other

fluoridated solutions (*i.e.*, 500 and 1100 ppm F), following a dose-response pattern according to CaGP concentrations (Table 1). The buffering effect of CaGP on biofilm has also been reported in studies in primates (Bowen 1969; Bowen 1972), which is possibly related to the formation of HPO_4^{2-} (Table 3). It is known that monohydrogen phosphate has a buffering effect (Aranha 2002, Dawes et al., 2015), and the higher concentrations of this compound are related to the higher pH values found after treatment with CaGP, mainly for 0.5 CaGP/F after sucrose exposure (Table 3). In addition, the presence of F interferes with the acidogenicity of *S. mutans*, as it alters the gene expression linked to glycosyltransferases and glycolysis, processes that lead to a decrease in pH (Pandit et al., 2017).

It is noteworthy that only biofilms treated with CaGP alone (*i.e.*, without F) did not show difference in pH values before and after exposure to sucrose (Table 1), which may be related to the high Ca values found in the biofilm fluid prior exposure to sucrose (Figure 1 B). The increase in Ca concentrations in the medium activates the autolysis of bacterial cells, causing cell lysis (Trombe et al., 1992), and thus, may have interfered with the production of acid by the biofilm.

In addition to pH, Ca and P concentrations in the biofilm fluid also interfere with the degree of saturation of the medium in relation to HA, protecting the tooth structure from demineralization (Pitts et al., 2017). The combination of CaGP and F was shown to lead to higher fluid saturation values in relation to HA (Table 2), what may be regarded to increases in Ca levels in the biofilm fluid caused by exposure to the treatments (Figure 1). It is known that the amount of Ca in the biofilm directly interferes in F uptake, given that the links between the bacterium and Ca is bidendatated, but becomes monodentate in the presence of the F, thus increasing the number of binding sites available for Ca (Rose et al., 1996). The above-mentioned mechanism seems to explain the present findings, since the higher the concentration of CaGP, the greater the concentration of Ca in the biomass, leading to higher concentrations of F in the biomass and, consequently, the lower concentration of F in the fluid (Figures 1 and 2).

The reduction of biofilm minerals after a cariogenic challenge has been demonstrated *in situ* (Cury et al., 1997) and *in vivo* (Bayrak et al. 2011). This pattern has been observed for most of the treatments analyzed, except for CaGP 0.125/F and CaGP 0.25/F, which led to an increase in both Ca and F in the biofilm biomass after exposure to sucrose (Figure 2). This fact may be related to the greater presence of CaGP in these biofilms. When biofilms are exposed to sucrose, F and Ca ions are released, but the

presence of CaGP in the fluid (a source of Ca ions) leads to absorption of F on the surface of microorganisms. This hypothesis is supported by the reduction of CaGP in the fluid after sucrose (Figure 1), despite this pattern was not observed when there was 0.5% CaGP in the treatment. This may be explained by the fact that CaGP interacts with F at appropriate molar ratios, and that when associated with 500 ppm F, the concentration of 0.25 CaGP leads to better results (Zaze et al., 2014a). As the pH drops, Ca linked to the bacteria is displaced by H +, which can reduce the driving force of tooth demineralization. This dynamic shows the importance of a bacterial calcium reservoir in the dynamics of the caries process (Leitão et al., 2018).

Another important point is that the reduction of F in the biofilm fluid after exposure to sucrose was lower in biofilms treated with CaGP+F thant for those treated with F alone (*e.g.*, reductions of 32.2% and 68.8%, respectively for CaGP 0.5/F and 500 ppm F). Given that the effectiveness of F as a cariostatic agent depends on its availability in the biofilm fluid during the cariogenic challenge (Rošin-Grget et al., 2013), the present data corroborate to the fact that therapies able to increase Ca levels in the dental biofilm are useful to to increase the cariostatic potential of F (Whitford et al., 2002).

The addition of CaGP to low- F toothpastes (do Amaral et al. 2013; Zaze et al., 2014a) and restorative materials (Santos et al., 2019) was shown to reduce enamel demineralization, what can be related to CaGP adsorption to the enamel surface, and to the increase in ionic activity of neutral species, such as $CaHPO_4^0$ and HF^0 in the dental biofilm (Cochrane et al., 2008, do Amaral, et al. 2013; Zaze et al., 2014a). The results found in the present study showed an increased formation of $CaHPO_4^0$ in the biofilm fluid, while the formation of HF was not different from the NC (Table 3). This lack of possible HF formation in the biofilm fluid must be related to the absence of H^+, since there was no acid production (Table 1) and the F being complexed in the biomass.

Despite the present study provided additional evidence on the mechanisms by which CaGP and F interfere in caries dynamics, limitations including the lack of a dental surface for ionic interaction and biofilm formation, the absence of continuous salivary flow, and use of a biofilm model that does not fully represent the complex oral microbiome. should be taken into account. Nevertheless, these limitations were necessary to minimize factors that would interfere with the data collected. Interestingly, despite the differences between *in vitro* and *in situ* conditions regarding biofilm formation and and sucrose challenges, Ca levels in the biofilm fluid were similar to a previous *in situ* study

(Nagata et al., 2017), where the values of 0.25CaGP/F did not differ from the values of 500 ppm F, after sucrose exposure.

5. Conclusions

Based on the above, it is possible to conclude that CaGP influenced a dual-species biofilm of *S. mutans* and *C. albicans*. Such activity is related to higher concentrations of F, Ca and P in the biofilm biomass, which acts as a reservoir for these components, and changes in the biofilm pH values. Thus, the use of this polyphosphate combined with F leads to changes in the biofilm (biomass and biofilm fluid) that can interfere in dental caries dynamic.

6. Acknowledgements

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Table 1. Mean (standard deviation) pH values of the biofilm after exposure to sucrose

	Groups								
	0.125 CaGP	**0.25 CaGP**	**0.5 CaGP**	**0.125 CaGP /F**	**0.25 CaGP /F**	**0.5 CaGP /F**	**Control**	**500 ppm F**	**1100 ppm F**
no-sucrose	5.77 ^{Aa} (0.13)	6.06 ^{Ab} (0.25)	6.62 ^{Ac} (0.14)	7.21 ^{Ab} (0.10)	7.63 ^{Ae} (0.08)	7.90 ^{Af} (0.18)	5.51 ^{Ag} (0.17)	6.42 ^{Ah} (0.17)	6.64 ^{Ac} (0.20)
Sucrose	5.71 ^{Aa} (0.12)	5.96 ^{Ab} (0.10)	6.63 ^{Ac} (0.21)	6.59 ^{Bc} (0.17)	7.11 ^{Bd} (0.10)	7.40 ^{Be} (0.15)	4.97 ^{Bf} (0.10)	5.97 ^{Bb} (0.12)	6.23 ^{Bg} (0.03)

Distinct upper- and lower-case letters indicate statistical difference between experimental conditions (no sucrose and sucrose), and among the treatment groups, respectively (Fisher LSD's test; $p<0.05$).

Table 2. Degree of saturation values (standard deviation) in relation to hydroxyapatite (HA) and calcium fluoride (CaF_2) in the biofilm fluid, before (no sucrose) and after (sucrose) exposure to sucrose, according to the groups

Groups	***Degree of Saturation***			
	HA		CaF_2	
	no sucrose	sucrose	no sucrose	sucrose
0.125 CaGP	-1.26 ^{Aa} (1.44)	-4.77 ^{Ba} (0,80)	-3.61^{Aa} (0.18)	-4.04 ^{Ba} (0.36)
0.25 CaGP	2.10 ^{Ab} (1.61)	-1.70 ^{Bb} (0.49)	-3.79 ^{Ab} (0.10)	-3.82 ^{Ab} (0.21)
0.5 CaGP	5.59 ^{Ac} (0.75)	3.27 ^{Bc} (1.25)	-3.36 ^{Ac} (0.28)	-3.73 ^{Bb} (0.07)
0.125 CaGP/F	5.70 ^{Ac} (0.56)	0.05 ^{Bd} (1.08)	2.44 ^{Ad} (0.10)	1.23 ^{Bc} (0.10)
0.25 CaGP/F	6.61 ^{Ad} (0.50)	2.20 ^{Be} (0.70)	1.86 ^{Ae} (0.14)	1.19 ^{Bc} (0.11)
0.5 CaGP/F	9.85 ^{Ae} (0.80)	6,18 ^{Bf} (0.68)	2.08^{Af} (0.09)	1.52 ^{Bd} (0.14)
Control	-3,34 ^{Af} (1.10)	-11.20 ^{Bg} (0.71)	-3.71^{Aab} (0.19)	-4.36 ^{Be} (0.22)
500 ppm F	1,94 ^{Ab} (1,20)	-4.78 ^{Ba} (0.87)	3.25^{Ag} (0.10)	1.85 ^{Bf} (0.09)
1100 ppm F	0,78 ^{Ag} (1,29)	-3,83 ^{Bh} (0.43)	2.98^{Ah} (0.06)	2.05 ^{Bg} (0.05)

Distinct upper- and lower-case letters indicate statistical difference between experimental conditions (no sucrose and sucrose) and among the treatment groups, respectively (Fisher LSD's test; $p<0.05$).

Table 3. Possible formations of $CaHPO_4^0$, HPO_4^{-2} and HF^0 in the biofilm fluid before and after contact with the sucrose

Groups	Ion activity (mmol/L)					
	$CaHPO_4^0$		HPO_4^{2-}		HF^0	
	no-sucrose	**sucrose**	**no-sucrose**	**sucrose**	**no-sucrose**	**Sucrose**
0.125 CaGP	1.90×10^{-2} (0.01) ^{Aa}	2.13×10^{-3} (0.00) ^{Ba}	1.74×10^{-2} (0.00) ^{Aa}	5.50×10^{-3} (0.00) ^{Bab}	7.67×10^{-6} (0.00) ^{Aa}	9.36×10^{-6} (0.00) ^{Aa}
0.25 CaGP	6.33×10^{-2} (0.03) ^{Ab}	6.15×10^{-3} (0.00) ^{Ba}	3.79×10^{-2}(0.03) ^{Ab}	7.50×10^{-3} (0.00) ^{Aa}	3.05×10^{-6} (0.00) ^{Aa}	4.58×10^{-6} (0.00) ^{Aa}
0.5 CaGP	1.19×10^{-1} (0.02) ^{Ac}	3.00×10^{-2} (0.00) ^{Bb}	5.94×10^{-2} (0.01) ^{Ac}	2.67×10^{-2} (0.01) ^{Bc}	1.07×10^{-6} (0.00) ^{Aa}	9.44×10^{-7} (0.00) ^{Aa}
0.125 CaGP/F	5.05×10^{-2} (0.01) ^{Ad}	7.55×10^{-3} (0.00) ^{Ba}	9.12×10^{-2} (0.01) ^{Ad}	2.88×10^{-2} (0.01) ^{Bc}	3.95×10^{-4} (0.00) ^{Aa}	6.35×10^{-4} (0.00) ^{Aa}
0.25 CaGP/F	3.50×10^{-2} (0.01) ^{Ae}	7.53×10^{-3} (0.00) ^{Ba}	8.59×10^{-2} (0.01) ^{Ad}	2.64×10^{-2} (0.00) ^{Bc}	9.27×10^{-5} (0.00) ^{Aa}	1.66×10^{-4} (0.00) ^{Aa}
0.5 CaGP/F	7.83×10^{-2} (0.01) ^{Af}	3.14×10^{-2} (0.00) ^{Bb}	5.67×10^{-2} (0.01) ^{Ac}	3.92×10^{-2} (0.00) ^{Bd}	3.68×10^{-5} (0.00) ^{Aa}	7.58×10^{-5} (0.00) ^{Aa}
Control	7.49×10^{-3} (0.00) ^{Ag}	1.68×10^{-4} (0.00) ^{Aa}	9.66×10^{-3} (0.00) ^{Aa}	5.04×10^{-4} (0.00) ^{Aa}	1.57×10^{-5} (0.00) ^{Aa}	3.80×10^{-5} (0.00) ^{Aa}
500 ppm F	3.12×10^{-2} (0.01) ^{Ae}	1.37×10^{-3} (0.00) ^{Ba}	5.23×10^{-2} (0.01) ^{Ac}	5.74×10^{-3} (0.00) ^{Bab}	6.34×10^{-3} (0.00) ^{Ab}	5.60×10^{-3} (0.00) ^{Ab}
1100 ppm F	1.41×10^{-2} (0.01) ^{Aag}	1.72×10^{-3} (0.00) ^{Ba}	7.06×10^{-2} (0.02) ^{Ab}	1.22×10^{-2} (0.00) ^{Bb}	4.97×10^{-3} (0.00) ^{Ac}	4.75×10^{-3} (0.00) ^{Ac}

Distinct upper- and lower-case letters indicate statistical difference between experimental conditions (no sucrose and sucrose) and among the groups (Fisher LSD's test; $p<0.05$).

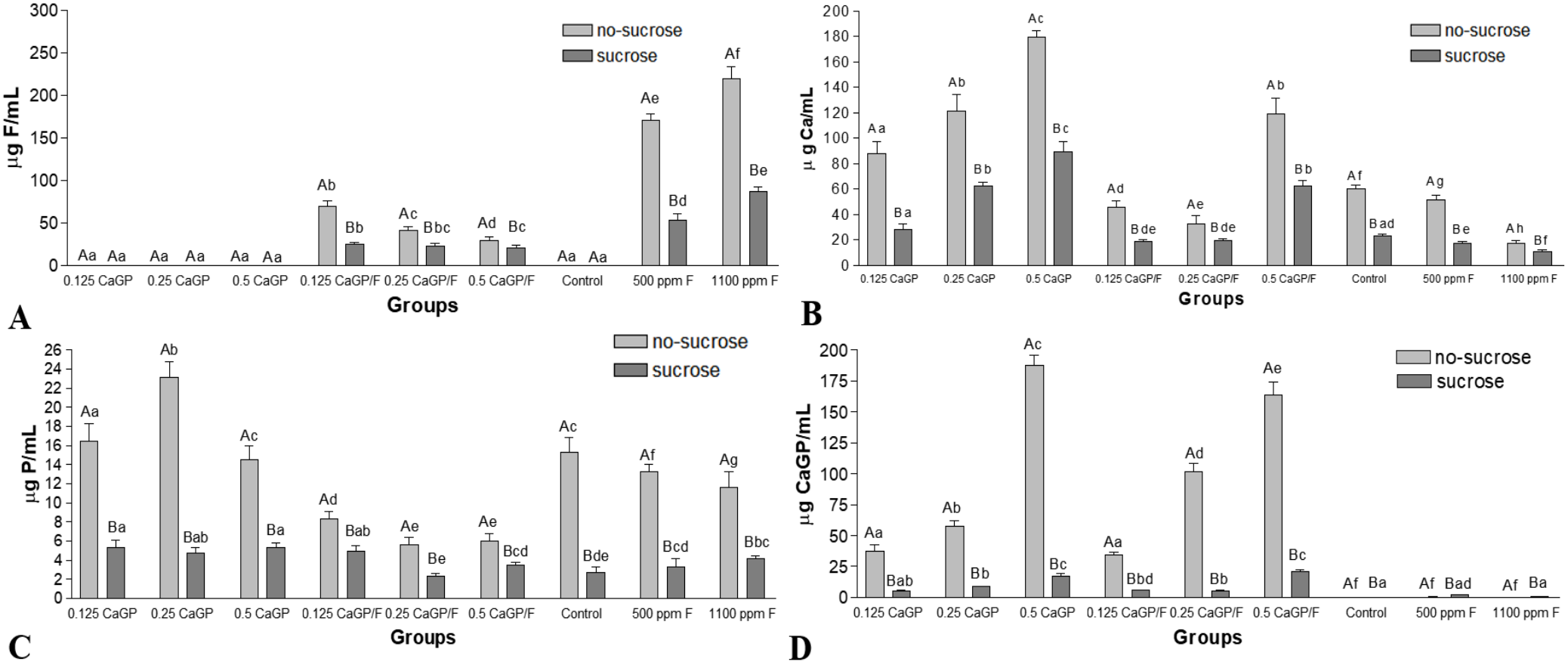

Figure 1. Mean values of F (A), Ca (B), P (C) and CaGP (D) in µg/mL of biofilm fluid, before and after contact with sucrose. Distinct upper- and lower-case letters indicate statistical difference between experimental conditions (no sucrose and sucrose) and among the groups (Fisher LSD's test; $p<0.05$).

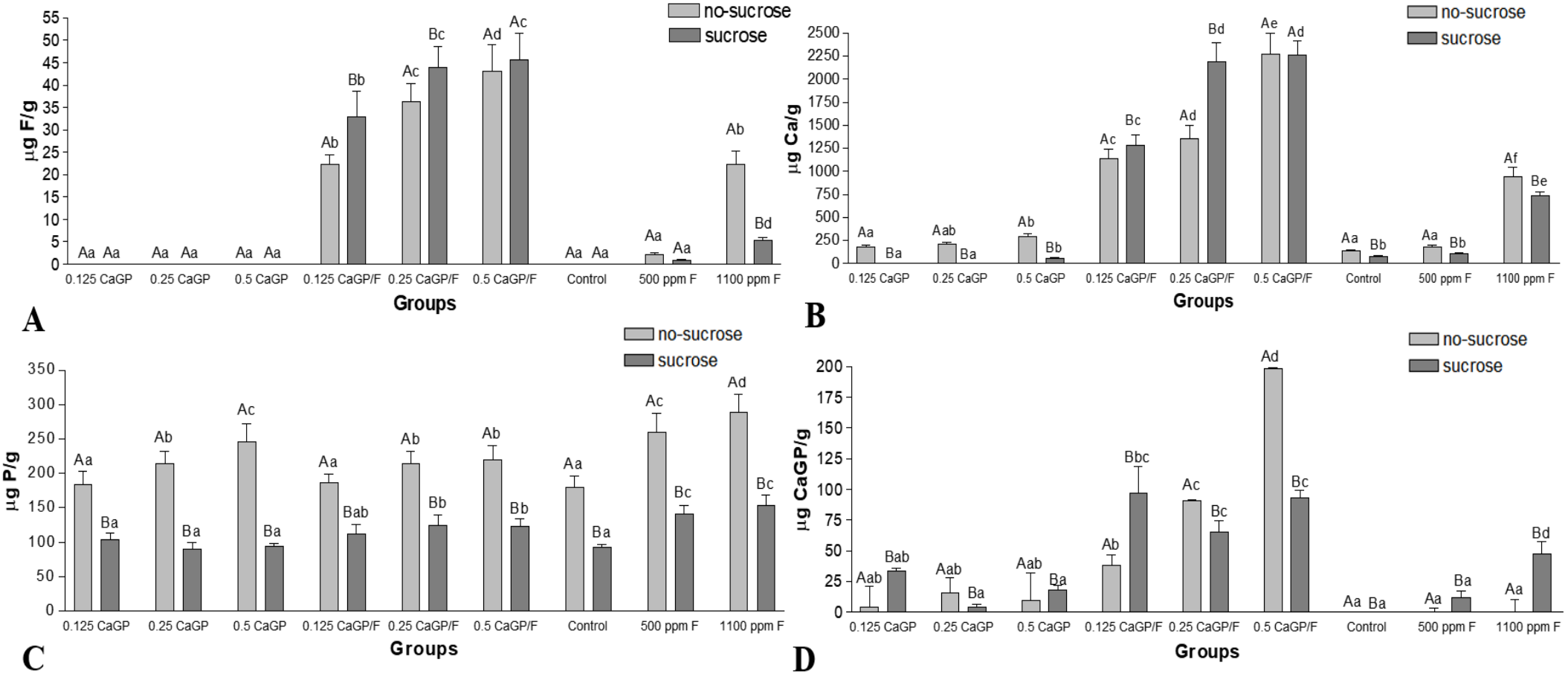

Figure 2. Mean values of F(A), Ca(B), P(C) and CaGP (D) in µg/g of biofilm biomass, before and after contact with sucrose. Distinct upper- and lower-case letters indicate statistical difference between experimental conditions (no sucrose and sucrose) and among the groups (Fisher LSD's test; $p<0.05$).

APÊNDICE

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ANEXO A

Normas periódico Biofouling

Preparing Your Paper

Structure

Your paper should be compiled in the following order: title page; abstract; keywords; main text introduction, materials and methods, results, discussion; acknowledgments; declaration of interest statement; references; appendices (as appropriate); table(s) with caption(s) (on individual pages); figures; figure captions (as a list).

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ANEXO B

Normas periódico The International Journal of Biochemistry & Cell Biology

Article structure

The structure of the article differs per article type. Please refer to the "Structure requirements for IJBCB articles" for detailed instructions for each article type.

Subdivision - numbered sections Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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