Thayse Yumi Hosida

Efeito do hexametafosfato de sódio, associado ou não ao fluoreto, no biofilme misto contendo *Streptococcus mutans* e *Candida albicans*

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Thayse Yumi Hosida

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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 Ciência Odontológica – Área Saúde Bucal da Criança.

Orientador: Prof. Tit. Alberto Carlos Botazzo Delbem
Coorientador: Prof. Ass. Juliano Pelim Pessan.
Coorientador: Prof. Dr. Douglas Roberto Monteiro

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Dedicatória

Dedico este trabalho

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Resumo Geral

Hosida, TY. **Efeito do hexametafosfato de sódio, associado ou não ao fluoreto, no biofilme misto contendo *Streptococcus mutans* e *Candida albicans.*** 2018. 111 f. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2018.

RESUMO

O presente estudo teve como objetivo verificar o efeito do hexametafosfato de sódio (HMP), associado ou não ao fluoreto (F), sobre a composição orgânica, inorgânica e no pH do biofilme mistos de *S. mutans* e *C. albicans* formados *in vitro*. Para todos os estudos, os biofilmes foram formados em poços de placas de microtitulação, colocando uma suspensão ($1x10^7$ células/mL *C. albicans* + $1x10^8$ células/mL *S. mutans*) em saliva artificial suplementada com sacarose (0,4%), a qual tinha metade de seu conteúdo renovada a cada 24 horas. Os biofilmes foram tratados três vezes (72, 78 e 96 horas de formação), por um minuto, com soluções contendo HMP (0.25, 0.5 ou 1%) com ou sem 500 ppm F, além de soluções contendo 500 e 1100 ppm F. A saliva artificial foi utilizada como controle negativo. Para o estudo microbiológico, após o terceiro tratamento foram realizados os testes de quantificação de células cultiváveis (CFU), biomassa total (teste colorimétrico de cristal violeta – CV), atividade metabólica (redução de XTT) e quantificação dos componentes da matriz extracelular (proteína, carboidrato e ácidos nucleicos). 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 ($p < 0.05$). O HMP apresentou efeito redutor principalmente na biomassa, metabolismo e nos componentes da matriz extracelular do biofilme. Biofilmes formados por 96 h formam expostos a três diferentes concentrações de sacarose (10, 20 ou 30%) durante 1, 3 ou 5 min. O pH foi medido antes da exposição à sacarose, imediatamente após sua remoção e após 1, 3, 5 e 10 min após a retirada da sacarose. Os resultados foram submetidos à análise de variância a três critérios, seguida pelo teste Fisher LSD ($p < 0.05$). O biofilme exposto a solução de sacarose a 20% por 3 min exibiu padrão de alteração de pH semelhante ao observado *in vivo*. Para o estudo da concentração de F, Ca, e Pi, após o período de tratamento, estes foram analisados no biofilme total e no fluido do biofilme após a mensuração do pH do biofilme. Em outro conjunto de experimentos, após o terceiro tratamento (96 h de formação de biofilme) 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 Pi tanto na biomassa como no fluido do biofilme. Os dados foram submetidos à análise de variância a dois critérios, seguida pelo teste de Fisher LSD ($p < 0.05$). O tratamento com HMP aumentou a concentração de F e Pi no fluido do biofilme antes da exposição à sacarose, além de manter o pH do meio mais próximo do neutro, mesmo após a exposição do biofilme à sacarose. Assim, é possível concluir que o HMP interfere na biomassa, metabolismo, composição orgânica e inorgânica, bem como no pH do biofilme testado.

Palavras-chaves: Fosfatos, Flúor, Biofilmes, *Streptococcus mutans* e *Candida albicans*

Abstract

Hosida, TY. **Effect of sodium hexametaphosphate, associated or not with fluoride, on mixed biofilm containing *Streptococcus mutans* e *Candida albicans***. 2018.111 f. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2018.

ABSTRACT

The aim of the present study was to verify the effect of sodium hexametaphosphate (HMP), associated or not to fluoride (F), on the inorganic, organic composition and pH of the mixed biofilm of *S. mutans* and *C. albicans*, formed *in vitro*. For all studies, the biofilms were formed in wells of microtiter plates by placing a suspension (1 x 10^7 cells/mL *C. albicans* + 1x10^8 cells/mL *S. mutans*) in artificial saliva supplemented with sucrose (0,4%), which had half of its content renewed every 24 hours. Biofilms were treated three times (72, 78 and 96 hours of formation), for one minute, with solutions containing HMP (0.25, 0.5 or 1%) with or without 500 ppm F, as well as solutions containing 500 and 1100 ppm F. Artificial saliva was used as a negative control. For the microbiological study, the following tests were performed: quantification of cultivable cells (UFC), total biomass (colorimetric crystal violet test - CV), metabolic activity (XTT reduction) and quantification of matrix components (protein, carbohydrate and nucleic acid). 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 ($p < 0.05$). HMP showed a reducing effect mainly on the biomass, metabolism and components of the extracellular matrix of the biofilm. Biofilms formed for 96 h were exposed to three different concentrations of sucrose (10, 20 or 30%) for 1, 3 or 5 min. The pH was measured before exposure to sucrose, immediately after its removal and after 1, 3, 5 and 10 min after removal of the sucrose. The results were submitted to 3-way analysis of variance, followed by the Fisher LSD test ($p < 0.05$). The biofilm exposed to 20% sucrose solution for 3 min exhibited a pattern of pH change similar to that observed *in vivo*. For the study of the concentrations of F, Ca, and Pi, these ions were analyzed in the total biofilm and in the biofilm fluid after treatment with the test solutions and after the pH measurement of the biofilm. In another set of experiments, after the third treatment (96 h of biofilm formation), the biofilms were exposed for 3 minutes to a 20% sucrose solution. This was removed and after 1 minute the biofilms

were collected, and the pH of the medium and F, Ca, and Pi concentrations were determined both in the biomass and in the biofilm fluid. The data were submitted to two-way analysis of variance, followed by Fisher LSD's test ($p < 0.05$). Treatment with HMP increased F and Pi concentration of the biofilm fluid prior sucrose exposition, and maintained the pH of the medium close to neutral values even after exposure of the biofilm to sucrose. Thus, it is possible to conclude that HMP interferes in the biomass, metabolism, organic and inorganic composition and the pH of the biofilm tested.

Keywords: Phosphates, Fluoride, Biofilms, *Streptococcus mutans* and *Candida albicans.*

Lista de figuras

LISTA DE FIGURAS

Capítulo 1

Figure 1 Logarithm of colony-forming units per cm^2 for *S. mutans* (a) and *C. albicans* (b) biofilms, and absorbance values per cm^2 obtained for the total biomass (c) and metabolic activity (d) quantification assays. NC: negative control (untreated biofilms). Error bars denote the standard deviations of the means. Different upper-case letters symbolize statistical differences among the groups ($p < 0.05$). 54

Capítulo 2

Figure 1 Mean pH values determined after exposure of the mixed biofilm of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175 to 10% sucrose (A), 20% sucrose (B) and 30% sucrose (C), as a function of the exposure time to sucrose and time of pH determination. Baseline: pH values determined before biofilm exposure to sucrose; t0: pH determined immediately after sucrose removal; t1, t3, t5 and t10: pH determined at 1, 3, 5 and 10 minutes after sucrose removal, respectively. Different capital letters between parentheses denote significant differences among exposure times to sucrose within the same sucrose concentration (Stand time). Different lowercase letters represent significant differences among pH values at each individual graph, as well as among sucrose concentrations. Vertical bars represent the standard deviation of the means (Student-Newman-Keuls, $p < 0.05$, $n = 3$). 67

Capítulo 3

Figure 1 Mean values of F (A), Ca (B), P (C) and HMP (D) in μg / mL of biofilm fluid, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). 89

Figure 2 Mean values of F (A), Ca (B), P (C) and HMP (D) in μg / mL of biofilm biomass, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). 90

Figure 3 Mean values of Ca (A), HMP (B), in μg / mL after cell lysis, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposition. 91

Figure 4 Possible formations of $CaHPO_4^0$ (A), HPO_4^{-2} (B), CaF^+ (C), HF^0 (D) in the biofilm fluid before and after contact with the sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). 92

Lista de tabelas

LISTA DE TABELAS

Capítulo 1

Table 1. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (CFM) and minimum bactericidal concentration (MBC) concentrations of HMP against the strains tested (n=3). 52

Table 2. Mean values (SD) of each matrix component of dual-species biofilms obtained after treatment with different concentrations of HMP, associated or not to F. 53

Capítulo 3

Table 1. Mean values (standard deviation) of pH of the biofilm after contact with sucrose. Different upper letters symbolize a statistical difference between groups ($p < 0.05$). 87

Table 2 Degree of saturation values (SD) in relation to hydroxyapatite (HA) and calcium fluoride (CaF_2) from biofilm fluid, before (no sucrose) and after (sucrose) contact with sucrose according to the groups. 88

Sumário

SUMÁRIO

INTRODUÇÃO GERAL 31

Capítulo 1 – **Effect of sodium hexametaphosphate, associated or not to fluoride, on mixed biofilms**

ABSTRACT 37

INTRODUCTION 38

MATERIALS AND METHODS 39

RESULTS 42

DISCUSSION 43

REFERENCES 48

Capítulo 2 – **pH changes of mixed biofilms of *Streptococcus mutans* and *Candida albicans* after exposure to sucrose solutions *in vitro***

ABSTRACT 57

INTRODUCTION 58

MATERIALS AND METHODS 59

RESULTS 60

DISCUSSION 61

REFERENCES 64

Capítulo 3 – **Sodium hexametaphosphate and fluoride affect F, Ca, Pi and pH of dual-species biofilms after sucrose exposure**

ABSTRACT 70

INTRODUCTION 71

MATERIALS AND METHODS 72

RESULTS 75

DISCUSSION 77

REFERENCES 82

ANEXOS 95

Introdução Geral

INTRODUÇÃO GERAL

A cavidade bucal é composta por tecidos duros e moles, os quais propiciam um ambiente favorável para a formação de biofilmes polimicrobianos (Jakubovics & Kolenbrander, 2010). A relação entre diferentes microrganismos do biofilme pode aumentar a sua resistência (Jakubovics & Kolenbrander, 2010) e o desequilíbrio dessas comunidades pode exacerbar a patogenicidade das espécies e contribuir para o surgimento de doenças orais relacionadas ao biofilme, como a cárie dentária. A cárie é uma doença biofilme-açúcar dependente (Sheiham & James, 2015), ocasionada pela ação de ácidos produzidos por bactérias a partir da fermentação de carboidratos da dieta, os quais progressivamente levam a perda de minerais do dente, decorrente da constante queda de pH (Cummins & Bowen, 2006).

Um dos principais agentes etiológicos dessa doença é a bactéria Gram-positiva *Streptococcus mutans*, devido à sua capacidade de colonizar a superfície dentária, metabolizar carboidratos, produzir matriz extracelular e ácido láctico, além de ter a capacidade de crescer e se multiplicar em ambiente ácido (Marsh & Martin, 2009; Lamont *et al*., 2006), características que conferem alto grau de virulência (Loesche, 1986; Krzysciak *et al*., 2014). Além de bactérias, o biofilme dental é composto por outros microrganismos, dentre os quais se destacam *Candida albicans*, que é o fungo mais comumente encontrado na cavidade oral (Nikawa *et al*., 2003). A presença de *C. albicans* é um importante fator na progressão da cárie dentária, uma vez que contribui em sua patogênese em crianças cárie-ativas (de Carvalho *et al*., 2006, Klinke *et al*., 2009), pois possui enzimas proteolíticas que realizam a degradação do colágeno (Pereira *et al*., 2018). Além disso, tem sido relatado que *C. albicans* interage com *S. mutans*, acentuando as características de virulência dos microrganismos cariogênicos (Raja *et al*., 2010; Metwalli *et al*., 2013).

Quando organizados na forma de biofilmes, os microrganismos se apresentam embebidos em uma matriz extracelular composta por glicoproteínas e polissacarídeos (ten Cate *et al*., 2009). Inicialmente, várias adesinas de bactérias 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 dentária, como resultado da produção de exopolissacáridos (glucanos), por meio da atividade das enzimas glicosiltransferases (GTFs). Assim, o amadurecimento do biofilme promove um metabolismo mais eficiente da sacarose pelo

o *S. mutans*, levando à produção de ácido láctico (Marsh & Martin, 2009; Lamont *et al*., 2006). Este, por sua vez, causa quedas de pH, o que acarreta um desequilíbrio no estado de saturação dos minerais do fluido do biofilme em relação à estrutura dentária.

O biofilme dental tem a capacidade de reter íons Ca^{2+} e F^{-} na forma de depósitos minerais (Kaufman & Kleinberg, 1976), ligados a grupamentos aniônicos das paredes das bactérias (Rose *et al.* 1993; 1996) e ligados nas proteínas da matriz do biofilme (Gao *et al,*. 2001). Assim, o biofilme dental atua como um reservatório de íons que podem ser liberados para o fluido do biofilme durante quedas de pH, portanto interferindo em seu grau de saturação, reduzindo a perda mineral do dente. Neste sentido, há evidência de que ocorre um aumento significativo na concentração de $Ca2^{+}$ no fluido do biofilme após um desafio cariogênico (Margolis & Moreno, 1992; Rankine *et al.* 1996; Tanaka & Margolis, 1999), o qual resulta da liberação a partir destes reservatórios.

Além do F e Ca^{2+}, as concentrações de fosforo (Pi) no biofilme dental (biomassa e fase fluida) exercem papel fundamental nos processos de desmineralização e remineralização da estrutura dentária, visto que a concentração destes íons no biofilme dental apresenta uma relação inversa com a incidência de cárie (Shaw *et al*., 1983), possivelmente devido à liberação destes íons para o fluido do biofilme (Buzalaf *et al*., 2011). Quanto ao processo de remineralização, este pode ocorrer através da precipitação de fosfatos de cálcio ou pelo crescimento dos cristais de esmalte remanescentes através do Ca e P presentes na saliva (Buzalaf *et al*., 2011).

Os dentifrícios fluoretados têm contribuído substancialmente para redução na prevalência da cárie dentária (Pessan *et al*., 2011), visto que sua utilização associa a remoção ou desorganização periódica do biofilme dental com as propriedades cariostáticas do fluoreto (F) (Pessan *et al*., 2006; Tenuta *et al*., 2009). Sendo assim, o uso frequente dos dentifrícios promove a manutenção de concentrações elevadas de F na saliva durante o dia, o que é responsável por seu efeito preventivo e terapêutico. Em acréscimo, a formação de produtos da reação esmalte dentina com F, formando o mineral fluoreto de cálcio (CaF_2), também é responsável pelos efeitos supracitados, uma vez que o depósito destes reservatórios no biofilme dental e em lesões de cárie iniciais é capaz de interferir na progressão das mesmas (Buzalaf *et al*., 2011).

Apesar do declínio na incidência e prevalência da cárie dentária observado em vários países, tem havido uma preocupação crescente em potencializar o efeito do F como estratégia para indivíduos severamente acometidos pela doença (Vogel *et al*.,

2008). Neste sentido, medidas que visem a aumentar os reservatórios iônicos intrabucais devem estar fundamentadas em estudos que avaliam a capacidade destes íons em permanecer por um longo período de tempo no biofilme dental. Dentre as estratégias disponíveis, a suplementação de veículos fluoretados com sais de fosfato tem sido uma possibilidade para se aumentar a efetividade do F, uma vez que estes parecem agir como uma barreira parcial aos ácidos bacterianos devido a sua afinidade com a superfície do esmalte (da Camara *et al*., 2014).

Estudos *in vitro* demonstraram que dentifrícios com concentração reduzida de F suplementados com hexametafosfato de sódio (HMP) apresentam efeito semelhante ao de um dentifrício convencional (1100 ppm F) sobre a desmineralização do esmalte (da Camara *et al.,* 2014). Os efeitos da associação do HMP e F dependem da proporção molar entre estes compostos (da Camara *et al*., 2015, da Camara *et al*., 2016), de forma que a concentração ideal de HMP a ser utilizada é de 1% em formulações de dentifrício contendo 1100 ppm F (da Camara *et al.*, 2015, da Camara *et al*., 2016). Quanto aos efeitos sobre o biofilme dental, demonstrou-se que a associação de 1% de HMP a 1100 ppm de F promoveu a menor retenção de Ca e maior retenção de F quando comparado ao grupo controle (1100 ppm F), enquanto os valores para P foram semelhantes entre os tratamentos. Em acréscimo, esta associação promoveu uma redução significativa na concentração de polissacarídeos extracelulares comparado ao controle negativo (placebo), porém não diferenciou estatisticamente do grupo tratado com 1100 ppm F (da Camara *et al.,* 2015).

Com base no exposto, a associação entre HMP e F apresenta efeito anticárie sobre a desmineralização do esmalte, porém o efeito desta associação no biofilme dental ainda é escasso e contraditório, visto que não foi avaliado o fluido do biofilme e quando analisado o biofilme ocorreu um aumento de F e diminuição da concentração de Ca após desafio cariogênico (da Camara *et al.,* 2015). Devido à capacidade quelante do HMP, ligações com íons presentes no biofilme e na parede dos microrganismos poderiam interferir na disponibilidade de íons como o Ca^{2+}, pois estes estariam ligados ao HMP. Este aspecto reforça a necessidade de estudos adicionais avaliando os efeitos do F, Ca e do HMP 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 dinâmica da cárie dentária.

Portanto, seria interessante conduzir um estudo *in vitro* avaliando os efeitos da associação entre F e HMP sobre a composição e metabolismo de um biofilme misto de *S. mutans* e *C. albicans*, sobre a retenção de F, P e Ca não somente no biofilme total, mas também no fluido do biofilme antes e após a exposição à sacarose e sobre o pH deste biofilme.

Para abordar o tema proposto, o estudo será apresentado em três capítulos distintos, conforme descrito abaixo:

- Capítulo 1: **"Effect of sodium hexametaphosphate, associated or not to fluoride, on mixed biofilms"**
(artigo submetido ao periódico Future Microbiology);
http://www.futuremedicine.com/userimages/ContentEditor/1340638225103/fm_author_guidelines.pdf

- Capítulo 2: **"pH changes of mixed biofilms of *Streptococcus mutans* and *Candida albicans* after exposure to sucrose solutions *in vitro*"**
(artigo aceito no periódico Archives of Oral Biology);

- Capítulo 3: **"Sodium hexametaphosphate and fluoride affect F, Ca, Pi and pH of dual-species biofilms after sucrose exposure"**
(artigo formatado de acordo com as normas do periódico Journal of Dentistry).
https://www.elsevier.com/journals/journal-of-dentistry/0300-5712?generatepdf=true

Capítulo 1

Effect of sodium hexametaphosphate and fluoride on dual-species biofilms

Activity of sodium hexametaphosphate on biofilms

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Effect of sodium hexametaphosphate and fluoride on dual-species biofilms

Abstract

Aim: To evaluate the effect of sodium hexametaphosphate (HMP), associated or not to fluoride (F), on dual-species biofilms. **Materials & Methods:** HMP minimum inhibitory concentration (MIC) was determined. After, 72-h biofilms were treated with HMP solutions at 0.25, 0.5 and 1%, combined or not with 500 ppm F, and analyzed by quantification of colony-forming units (CFUs), metabolic activity, biomass and extracellular matrix components. **Results:** MIC of HMP was 0.187% (*Candida albicans*) and 0.093% (*Streptococcus mutans*). Treatments with HMP/F significantly reduced the CFUs of *S. mutans* compared to the negative control. HMP treatments also reduced biomass, metabolic activity and extracellular matrix compounds. **Conclusions:** HMP/F influenced CFU, biomass, metabolism and extracellular matrix of the dual-species biofilms analyzed.

Keywords: Biofilms. *Streptococcus mutans*. *Candida albicans*. Polyphosphates. Fluorides.

Introduction

The formation and persistence of biofilms on tooth surfaces is one of the main etiological factors to dental caries. Dental biofilm consists of a microbial community adhered to dental surfaces, immersed in an amorphous material named extracellular matrix. Around 70% of the total volume of the biofilm is constituted by microbial cells [1], while the remaining is composed of extracellular matrix, which wraps and ensures structural integrity to the bacterial mass [2]. Among the bacterial cells, the genus streptococcus is dominant, being *Streptococcus mutans* a pathogenic agent widely found in cavitated caries lesions [3]. Taking into account the polymicrobial nature of the oral biofilms, other species may also contribute to the development of dental caries, such as *Candida albicans* [4]. This fungus plays an important role in the processes of de- and re-mineralization of dental structures, having the ability to produce acids and proteolytic enzymes, which degrade collagen, contributing to the dentin cavity formation [4].

The association among microorganisms within pathogenic biofilms may contribute to resistance to conventional therapy [5], which has encouraged studies assessing alternative antimicrobial agents with potential to interfere in biofilm formation and its composition. Sodium Hexametaphosphate (HMP) is an inorganic cyclophosphate that presents antimicrobial activity by binding to cell wall of Gram-positive bacteria, increasing its permeability [6, 7, 8]. HMP has been assessed in fluoridated dentifrices, with remarkable synergistic effects on enamel demineralization [9].

In addition to the mineralizing properties, studies have shown that the association of inorganic phosphates with fluoride (F) in dentifrices decreased the production of extracellular polysaccharides (EPS) from biofilms exposed to sucrose in comparison to its counterpart without phosphate [10, 11]. Furthermore, the above-mentioned studies showed that the phosphate-containing toothpastes led to lower Ca and higher F uptake by the dental biofilm in comparison to 1100 ppm F [10, 11]. It is well established that EPS from biofilms are able to promote significant pH drop in the tooth/biofilm interface when exposed to fermentable carbohydrates [12, 13]. However, the literature presents few and conflicting [14] data on the role of inorganic phosphates associated to F in biofilms. It thus become evident that further studies are necessary to evaluate whether the effects of the association between fluoride and an inorganic phosphate are mostly related to the phosphate interaction with tooth enamel [9, 11, 15] providing a protective

barrier against demineralization [9], or it acts on biofilm formation and the resulting cell dynamic of the microorganisms.

Thus, the aim of this study was to assess the effect of HMP, associated or not to F, on dual-species biofilms of *S. mutans* and *C. albicans*, using different biofilm quantification assays (cultivable cells, total biomass, metabolic activity and extracellular matrix composition). The study's null hypothesis was that the association of HMP and F would not affect the parameters analyzed in comparison with treatment with F alone at the same concentration.

Materials and methods

Artificial saliva

The culture medium used for biofilm formation was sucrose-containing artificial saliva (AS), and its composition for 1 L of deionized water was based on the protocol recommended by Lamfon *et al*. (2003) [16], with modifications: 4 g of sucrose (Sigma-Aldrich), 2 g of yeast extract (Sigma-Aldrich), 5 g of bacteriological peptone (Sigma-Aldrich), 1 g of mucin type III (Sigma-Aldrich), 0.35 g of NaCl (Sigma-Aldrich), 0.2 g of $CaCl_2$ (Sigma-Aldrich) and 0.2 g of KCl (Sigma-Aldrich). The pH of the solution was adjusted with NaOH to 6.8.

Strains and growth conditions

Two strains from American Type Culture Collection (ATCC) were employed in this study: *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. For *C. albicans*, colonies previously cultured on Sabouraud dextrose agar (SDA; Difco, Le Pont de Claix, France) were suspended in 10 mL Sabouraud Dextrose broth (Difco) and aerobically incubated overnight at 120 rpm and 37°C. *S. mutans* colonies grown on Brain Heart Infusion agar (BHI Agar; Difco) were suspended in 10 mL BHI broth (Difco) and statically incubated overnight in 5% CO_2 at 37°C. Afterwards, fungal and bacterial cells were recovered by centrifugation (8000 rpm, 5 min) and the cell pellets, washed twice with 10 mL of 0.85% NaCl. The number of *Candida* cells was adjusted to 10^7 cells/mL in AS using a Neubauer counting chamber, while bacterial cells were spectrophotometrically (640 nm) adjusted to 10^8 cells/mL.

Preparation of HMP solutions containing or not F

HMP solutions were prepared by diluting the salt (Sigma-Aldrich) in sterile deionized water to achieve final concentrations of 0.25, 0.5 and 1%. Next, NaF (SigmaAldrich) was added to each HMP solution to achieve a concentration of 500 ppm F. HMP concentrations were based on previous studies assessing the protective effect of this salt in association with fluoride at 250 and 1100 ppm F against enamel demineralization [9,11]. Based on the optimum HMP:F ratio determined in the above-mentioned studies, HMP at 1% was calculated as the most suitable concentration to be used in association with 500 ppm F. Solutions with lower HMP concentrations (0.25 and 0.5%) were used to evaluate possible dose-dependent effects.

Determination of the minimum inhibitory concentration against planktonic cells

To establish the minimum inhibitory concentration (MIC) of HMP against *S. mutans* and *C. albicans*, the broth microdilution assay was employed following the criteria of the Clinical and Laboratory Standard Institute (CLSI) [17], as modified by Fernandes *et al.* [18]. Stock solutions (60%) of HMP were first diluted in geometric progression using deionized water, from 2 to 1024 times. Each previously obtained phosphate concentration was diluted (1: 5) in RPMI 1640 (Sigma-Aldrich) and BHI broth, respectively for *C. albicans* and *S. mutans*. After 48 h, the contents of each well (from the MIC endpoint) were plated in ASD (for *C. albicans*) or BHI agar (for *S. mutans*) to determine the Minimum Fungicide Concentration (MFC) and Minimum Bactericidal Concentration (MBC) of HMP against the strains tested. The assays were performed in triplicate on three independent occasions.

Biofilm formation and treatment with HMP associated or not to F

Dual-species biofilms were formed in flat-bottom 96-well microtiter plates (Costar, Tewksbury, USA). For this, 100 µL of each microbial suspension (2 x 10^7 cells/mL for *C. albicans* + 2 x 10^8 cells/mL for *S. mutans*) were added to the wells and the plates, incubated in 5% CO_2 at 37ºC for 72 h. The medium was renewed every 24 h by removing 100 µL and adding an equal volume of fresh AS. Subsequently, 72-h biofilms were treated twice a day (10:00 a.m. and 4:00 p.m.) for 1 min, followed by a further 1-min treatment on the next day (10:00 a.m.) [19], with three different concentrations of HMP associated or not to F: 0.25% (0.25HMP), 0.5% (0.5HMP), 1% (1HMP), 0.25HMP + 500 ppm F (0.25 HMP/F), 0.5 HMP + 500 ppm F (0.5 HMP/F) and 1HMP + 500 ppm F (1HMP/F). After the last treatment, AS was removed from the wells, and

the resulting biofilms were washed once with 0.85% NaCl to eliminate planktonic cells. Solutions containing 500 and 1100 ppm F (500F and 1100F) were used to assess possible dose-response effects, whereas AS devoid of HMP and F was considered as negative control (NC).

Biofilm quantification assays

Cultivable cells

The number of cultivable cells was assessed by enumeration of colony-forming units (CFUs), as previously detailed [18]. Briefly, the resulting biofilms after treatment were resuspended in 0.85% NaCl and scraped from the wells. Biofilm suspensions were then serially diluted (in 0.85% NaCl) and plated on CHROMagar *Candida* (Difco) and BHI agar supplemented with 7 μg/mL amphotericin B (Sigma-Aldrich) [18]. Agar plates were incubated during 24-48 h at 37°C, and the number of CFUs was expressed as Log_{10} CFU/cm^2.

Total biofilm biomass

Biofilm biomass was quantified by the crystal violet (CV) staining assay, as reported by Monteiro *et al*. [20]. Biofilms were fixed for 15 min at room temperature with 99% methanol (Sigma-Aldrich), stained during 5 min with 1% CV (Sigma-Aldrich), and destained by exposure to 33% acetic acid (SigmaAldrich). Absorbance values were read at 570 nm and represented as a function of the area of the wells (absorbance/cm^2). Wells containing AS without microbial cells were used as blanks.

Metabolic activity

The evaluation of the metabolic activity of the biofilm cells was performed by the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2Htetrazolium hydroxide (XTT; Sigma-Aldrich) reduction method [18]. In summary, XTT and phenazine methosulphate (Sigma-Aldrich) solutions were combined and pipetted into the wells. The microtiter plates were incubated (37°C, 120 rpm) for 3 h protected from light, and the absorbance values were measured at 490 nm (absorbance/cm^2). Blanks were processed as described for the total biomass assay.

Analysis of extracellular matrix composition

For this assay, dual-species biofilms were developed in the 6-well plates (Costar) containing 4 mL of the microbial suspension, as described above. After treatment with HMP solutions, biofilms were resuspended in 0.85% NaCl, scraped from the wells, and the liquid phase of the extracellular matrix was extracted by sonication (for 30s at 30 W), as detailed elsewhere [21]. The bicinchoninic acid method (Kit BCA; Sigma-Aldrich) was performed for protein determination of the extracellular matrix, using bovine serum albumin as standard [21], while the carbohydrate content was measured by the well-established method of Dubois *et al.* [22], using glucose as standard. For nucleic acids content, a volume of 1.5 µl of the liquid phase of the extracellular matrix was spectrophotometrically analysed (at 260 and 280 nm) in a Nanodrop Spectrophotometer (EONC Spectrophotometer of EONC, Biotek, USA) [23]. Protein, carbohydrate and nucleic acids values were expressed as mg/g of biofilm dry weight.

Statistical analysis

All microbiological experiments were carried out in triplicate, on three different days. The normality of the data was verified by Shapiro-Wilk's test, followed by one-way ANOVA and Fisher's LSD post-hoc test (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). All analyzes were performed with a significance level of 5%.

Results

Effect on planktonic cells

For *C. albicans* planktonic cells, the MIC and MFC values of HMP were 0.187 and 1.5%, respectively. *S. mutans* was more susceptible to HMP, with MIC and MBC values of 0.093 and 0.375%, respectively, (Table 1).

Biofilm quantification

After biofilm treatment with different HMP concentrations, associated or not to F, the groups treated only with HMP did not reduce *S. mutans* CFUs in comparison to the negative control ($p > 0.05$; Figure 1a). However, in association to F, significant reductions were observed in comparison to the negative control ($p < 0.001$; figure 1a). While HMP at 0.25 and 0.5% associated to F were similar to 500 ppm F regarding the CFU number ($p > 0.05$), 1HMP/F promoted the highest reductions in the number of CFUs, and this effect was statistically different from those found for 500 and 1100 ppm

F ($p < 0.05$; figure 1a). Fluoride concentration also interfered on this parameter, given thtat 1100 ppm F presented a higher decrease in CFUs compared to 500 ppm F ($p < 0.001$; figure 1a). Regarding the number of CFUs for *C. albicans*, no significant differences among the treatments were observed ($p=0.255$; figure 1b).

Regarding total biofilm biomass, all groups treated with HMP, associated or not to F, and 500 ppm F promoted significant reductions compared to the negative control (Figure 1c), despite no significant differences were found among the test groups and 500 ppm F (figure 1c). Biofilms exposed to 1100 ppm F and 1HMP/F were significantly different from each other (figure 1c).

As for biofilm's metabolism, HMP, associated or not to F, promoted significant reductions in comparison to negative control, 500 ppm F and 1100 ppm F (Figure 1d). Significant differences regarding metabolic activity were not observed in biofilms treated with solutions containing only F and the negative control (Figure 1d).

Extracellular matrix composition

All treatments were able to significantly decrease the amount of protein, carbohydrates and nucleic acids from the biofilm extracellular matrix in comparison to the negative control ($p < 0.001$; Table 2). Regarding protein, treatment with 0.25HMP/F and 0.5HMP/F led to significant reductions when compared to their counterparts without F. No significant differences were seen between the 1HMP/F and 1HMP groups, with the highest decrease in protein content being observed for the 0.5HMP/F group. For carbohydrate (Table 2), groups treated only with HMP did not differ from each other, but exhibited a higher decrease in comparison to their F-counterparts. The amounts of nucleic acids in the 0.25HMP and 1HMP groups were significantly lower than those found for their F counterparts. The highest decreases in the nucleic acids and carbohydrate contents were found for the 0.25HMP group. For biofilms treated with F alone, 1100 ppm F was more effective in reducing the components of the extracellular matrix in comparison to 500 ppm F, except for carbohydrates (Table 2).

Discussion

C. albicans and *S. mutans* are microorganisms that interact with each other and are able to form biofilms on different oral surfaces, contributing to the development of dental caries [24]. Taking into account that HMP associated to F enhances the protective

effects of dentifrices on enamel demineralization and considering that this phosphate alone presents antimicrobial activity [6, 7], the assessment of the association of this phosphate with F on oral biofilms could bring important contribution to the understanding of the benefits of this association in the caries dynamics. The present study demonstrated that HMP had a reducing effect on all parameters analyzed considering the dual-species biofilm, and that such effects were influenced by fluoride regarding some of the analyses, thus leading to the partial rejection of the study's null hypothesis.

HMP presents great affinity to metallic ions (Mg^{2+}, Ca^{2+}, K^{+}, Al^{+}, Fe^{3+}), forming ionic complexes [25, 26, 27]. This feature enables this phosphate to bind to Ca^{2+} and Mg^{2+} present in the microorganisms' cell wall, thus increasing cell permeability, what is related to HMP's antimicrobial activity. Nevertheless, while HMP solutions without F were not able to decrease the number of CFUs of mixed biofilms of *C. albicans* and *S. mutans*, MIC assays showed that HMP alone inhibited the growth of planktonic cells of *S. mutans* and *C. albicans* at low concentrations (0.093 and 0.187%, respectively). These results may be associated to the biofilms' virulence, which is higher in comparison to planktonic cells [28], therefore demanding higher concentrations to promote a significant effect. All HMP groups associated to F reduced the number of CFUs of *S. mutans* in comparison to the negative control, being that 1HMP/F showed the highest CFU decrease, reaching values significantly lower than 1100 ppm F, what may be associated to MBC (0.375%). Such effect was possibly due to a synergic action of F and HMP at a higher concentration, since the effect of this association (1HMP/F) promoted superior effects in comparison with treatment with F (550 and 1100 ppm F) or HMP separately. This synergistic effect is in line with in *in vitro* studies in which dentifrices with HMP and F presented a higher protective effect compared to the control groups on enamel demineralization [9, 11, 15]. Regarding *C. albicans*, no treatment inhibited the number of CFUs, what could be justified by the more complex cellular structure of yeasts compared with bacteria, what possibly hinders the action of HMP and F. In addition, the increased expression of drug efflux pumps, protective features of the extracellular matrix, and the existence of "persister" cells in the biofilms [29] may help to explain the resistance verified for *C. albicans*.

Regarding biomass quantification, all treatments significantly decreased the total biomass in relation to the negative control, except for 1100 ppm F. In addition, biofilms

treated with HMP solutions and their F counterparts were not significantly different. Thus, despite both HMP and F reduced the total biomass, the association of the two compounds had no additive or synergic effect on this parameter. Previous studies have suggested that the association between HMP and F may promote the retention of CaF^+ e Ca^{2+} ions on HMP, by Na^+ substitution in the HMP cyclic structure. Therefore, the chelating ability of HMP enables binding of Ca^{2+} and F^- available in the oral environment, in addition to HMP binding to anionic groups present in the bacterial cell wall. Based on the above, the effect of the association HMP/F on the microorganism could be attributed only to the phosphate, since F^- ions would be bound to Ca^{2+}, and consequently to HMP (as CaF^+).

The metabolic activity of the biofilms, on the other hand, was influenced by HMP without a synergistic effect between HMP and F, and with no dose-response effect of HMP. Furthermore, HMP groups promoted significant reductions in the metabolic activity when compared to the 500 ppm F and 1100 ppm F groups. These results might be explained by the chelating action of this phosphate [30], which favors its binding to the cell walls of Gram-positive bacteria, causing exchanges in bacterial metabolism [31]. The reduction in the biofilm metabolism may also be associated to the reduction in availability of metallic nutrients from the medium (saliva), which may be bound to the HMP.

It was recently shown that dentifrices containing HMP associated to F significantly decreased the amount of extracellular polysaccharides (EPS) of biofilms formed *in situ* in comparison to a placebo formulation, without significant differences compared with its counterpart without HMP [14]. The results of the present study corroborate with the aforementioned data, since all biofilms treated with HMP, associated or not to F, showed significant reductions in the amount of carbohydrate from extracellular matrix in comparison to the negative control. Interestingly, however, the association HMP/F promoted significant increases in EPS production in comparison with their counterparts without F, resulting in higher values than 500 ppm F. Although the reasons for such effects are not apparent, the simultaneous analysis of EPS data with those of XTT (*i.e.*, lack of effect of F alone) and especially CFU (*i.e.*, additive effect of F and HMP) suggest that the increase in EPS production caused by HMP/F might be associated with defense mechanisms of the biofilm caused by the stress induced by the treatment. The effective disintegration of compounds from extracellular matrix reduces glucan production, thus affecting a relevant virulence factor related to dental caries [32], once it

increases the effect of drugs on biofilms, reduces acid production, consequently affecting enamel demineralization [33]. From a clinical perspective, the decreases observed in Table 2 suggest an advantage of the compounds evaluated, considering that HMP associated or not to F could minimize the formation of acid niches in the biofilm, cell adhesion and aggregation, exchange of genetic information among microorganisms, as well as other relevant functions of the extracellular matrix.

Although the design of a dual-species biofilm does not reproduce the polymicrobial nature of the dental biofilm and does not mimic the ionic exchanges that occur among the oral fluids, biofilm and tooth surfaces, this model was necessary in order to isolate the study variables while minimizing confounding factors that could interfere in the results. The present results show that HMP influenced on several variables assessed, but the association with F did not produce synergistic or additive effects. Given that Ca, F and phosphate from the oral environment are able to bind to HMP, and that the resulting complex (HMP-ion) can also bind to anionic groups on the bacterial surface, it might be hypothesized that these complexes could be released due to acid production upon carioge/nic challenges, so that HMP, Ca, F and phosphate would become available in biofilm fluid. Future studies addressing this hypothesis could bring novel and important data for a deeper understanding on the mechanisms of action of HMP co-administered with F on caries dynamics.

Conclusions

HMP alone had a reducing effect mainly on the biomass, metabolism and extracellular matrix components of the mixed biofilm of *S. mutans* and *C. albicans*, with no dose-response relationship between HMP concentrations and all parameters assessed. Furthermore, the addition of F to the HMP solutions did not influence its effects on the biofilm. These findings bring new perspectives regarding the mechanisms by which HMP acts in dental biofilm.

Future perspective

The effects of HMP in the reduction of the biomass, metabolism and components of the extracellular matrix observed in the present study, along with previous data on the protective effect of HMP co-administered with F on enamel

demineralization, suggest that formulations containing these salts are promising alternatives for caries control. Studies assessing the effects of this combination upon cariogenic challenges, as well as the evaluation of formulations with different F/HMP concentrations may bring important insights into the mechanisms of action of this phosphate on caries dynamics.

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Executive Summary

- One of the main etiological agents of dental caries is *Streptococcus mutans*, a Gram-positive and facultative anaerobic bacterium, which is able to ferment several sugars, producing lactic acid. Furthermore, a significant association between *S. mutans* and *C. albicans* and early childhood caries has been reported.
- Due to the anticaries and antimicrobial effects of fluoride and sodium hexametaphosphate (HMP), respectively, it was hypothesized that the association between HMP and fluoride could result in enhanced antibiofilm activity.
- HMP alone had a reducing effect mainly on biomass, metabolism and extracellular matrix components of the dual-species biofilm of *S. mutans* and *C. albicans* tested in the present study.
- The association of HMP and F may be promising for dental applications in which both compounds are co-administered.

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Table 1. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (CFM) and minimum bactericidal concentration (MBC) of HMP against the strains tested (n=3)

Species	HMP	
	MIC (%)	MFC/MBC (%)
C. albicans ATCC 10231	0.187	1.5
S. mutans ATCC 25175	0.093	0.375

Table 2. Mean values (SD) of each component of the extracellular matrix of dual-species biofilms obtained after treatment with different concentrations of HMP, associated or not to F

Matrix component (mg/g of biofilm dry weight)	**Groups**								
	NC	**500 F**	**1100 F**	**0.25HMP**	**0.25HMP/F**	**0.5HMP**	**0.5HMP/F**	**1HMP**	**1HMP/F**
Proteins	25.34^{A} (1.26)	18.93^{B} (1.28)	12.42^{C} (1.69)	12.74^{C} (1.00)	11.90^{D} (1.40)	15.29^{D} (1.40)	11.09^{C} (1.89)	15.65^{D} (2.48)	14.54^{D} (1.24)
Carbohydrates	542.91^{A} (29.31)	168.17^{BDE} (16.94)	160.84^{BCE} (15.89)	149.60^{E} (17.88)	234.10^{C} (22.07)	144.41^{E} (15.77)	185.41^{D} (18.01)	152.72^{E} (14.79)	229.37^{C} (22.12)
Nucleic acids	13.08^{A} (1.97)	9.21^{B} (0.55)	7.85^{C} (0.67)	8.18^{C} (0.47)	10.84^{B} (0.97)	9.28^{B} (0.19)	9.15^{B} (1.55)	9.35^{B} (0.67)	12.44^{A} (1.03)

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

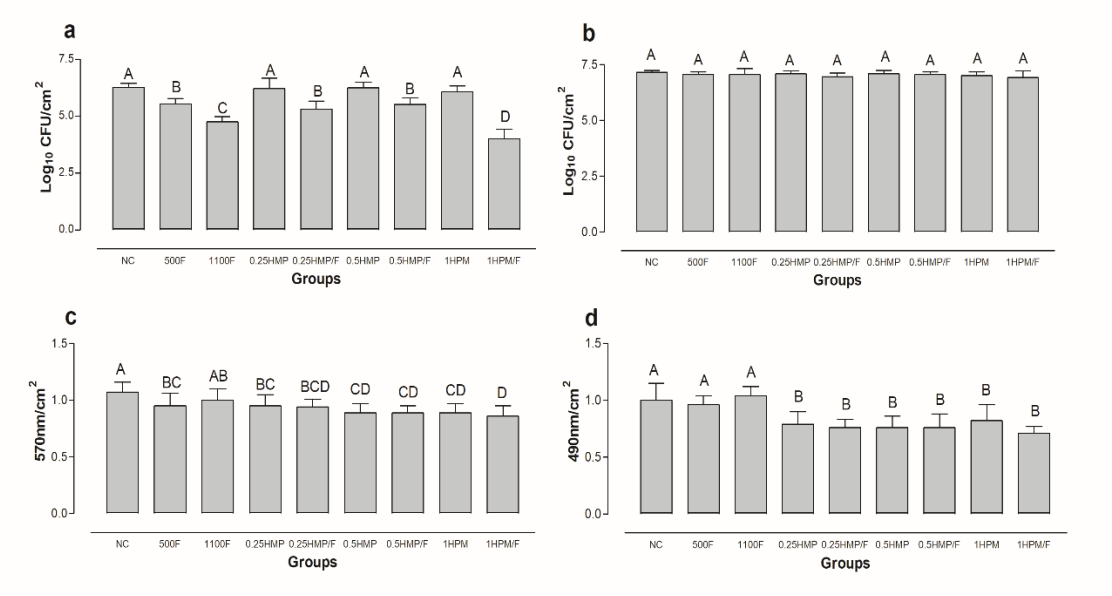

Figure 1. Logarithm of colony-forming units per cm^2 for *S. mutans* (a) and *C. albicans* (b) in dual-species biofilms, and absorbance values per cm^2 obtained for the total biomass (c) and metabolic activity (d) quantification assays. NC: negative control (untreated biofilms). Error bars denote the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

Capítulo 2

pH changes of mixed biofilms of Streptococcus mutans and Candida albicans after exposure to sucrose solutions in vitro

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Running title: *In vitro* reproduction of dental biofilm pH

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Abstract

Objective: This study aimed to standardize an *in vitro* experimental model able to reproduce the pH changes that occur in dental biofilm under *in vivo* conditions, using a mixed biofilm of *Streptococcus mutans* and *Candida albicans*.

Design: Biofilms were developed for 96 hours, and exposed to three different concentrations of sucrose (10, 20 or 30%) during 1, 3 or 5 minutes. The pH was measured before exposure to sucrose, immediately after its removal from the biofilms, and at 1, 3, 5 and 10 minutes after removal.

Results: Sucrose solutions at 10 and 20% required 1 minute to significantly reduce the biofilm pH, while for 30% sucrose a significant reduction was already seen immediately after its removal, even for the shortest exposure time. For an exposure of 3 minutes to 20% sucrose, the biofilm pH attained the critical value for hydroxyapatite dissolution when measured 1 minute after sucrose removal, followed by a recovery phase.

Conclusions: A mixed biofilm of *S. mutans* and *C. albicans* exposed to a 20% sucrose solution for 3 minutes exhibited a pattern of pH change similar to that observed *in vivo*, despite at a higher speed when compared to *in vivo* conditions.

Keywords: Biofilm model; *Candida albicans*; pH; *Streptococcus mutans*; sucrose.

1. Introduction

Dental caries is a biofilm- and sucrose-dependent disease (Sheiham & James, 2015), whose etiology, diagnosis, treatment, and control have been extensively studied over the last decades. *Streptococcus mutans* is the main pathogen related to this condition, mainly due to its capacity to ferment carbohydrates, survive in a low pH environment, and produce extra- and intracellular polysaccharides, which facilitate biofilm formation and adherence to dental surfaces (Kleinberg, 2002). Notwithstanding, in cases of early-childhood caries, *Candida albicans* is also found in the cariogenic biofilm (Falsetta et al., 2014), what contributes to its pathogenesis due to collagen degradation produced by proteolytic enzymes (Pereira et al., 2017). These microorganisms thrive better together and in presence of sucrose (Falsetta et al., 2014; Kim et al., 2017).

Caries lesions result from a mineral imbalance between tooth and biofilm on its surfaces (Fejerskov, 2004). In this sense, when the pH of the biofilm fluid decreases (pH < 5.5), it becomes undersaturated in relation to hydroxyapatite, resulting in dissolution of this mineral (Buzalaf, Pessan, Honório, & ten Cate, 2011). Regarding the effects of pH changes on dental biofilm, a time-course pH curve was designed *in vivo* (Stephan, 1944), presenting four distinct phases, as recently revised by Bowen (Bowen, 2013).

Due to the physiological complexity associated to the polymicrobial nature of the oral cavity, besides ethical issues involving clinical studies, there is an increasing interest in the development of laboratory models that mimic clinical conditions related to dental caries (Maske, van de Sande, Arthur, Huysmans, & Cenci, 2017). In this regard, new *in vitro* techniques have been developed to improve the knowledge about biofilm properties (Azeredo et al., 2017). The Stephan curve, for instance, was reproduced using an *in vitro* model of microcosm biofilm, in which the biofilm was exposed to 5 or 10% sucrose solutions by continuous flow, and its thickness was shown to affect the pH recovery (Sissons, Cutress, Faulds, & Wong, 1992).

Considering that the pH of a dual-species biofilm of *S. mutans* and *C. albicans* is neutralized after exposure to sucrose over time (Willems, Kos, Jabra-Rizk, & Krom, 2016), and that its behavior in relation to the Stephan curve remains unknown, the aim of the current study was to determine the sucrose concentration and its exposure time that would produce pH changes similar to those found *in vivo*, using a mixed biofilm model of the above-mentioned species.

2. Materials and methods

2.1. Experimental Design

Mixed biofilms of *S. mutans* and *C. albicans* were developed in sucrose-containing artificial saliva within 6-well plates, during 96 hours. After, biofilms were exposed to sucrose solutions at 10, 20 or 30%, which remained in contact with biofilms for 1, 3 or 5 minutes. Biofilm pH was measured before exposure to sucrose, immediately after its removal from the biofilms, and at 1, 3, 5 and 10 minutes after removal. Sucrose concentration, time of biofilm exposure to sucrose and time elapsed after exposure were considered as variation factors.

2.2. Artificial saliva

The composition of artificial saliva (for 1 L of deionized water) was based on the procedure described by Lamfon et al. (Lamfon, Porter, McCullough & Pratten, 2003), with minor modifications: 2 g of yeast extract (Sigma-Aldrich, St Louis, USA), 5 g of bacteriological peptone (Sigma-Aldrich), 1 g of mucin type III (partially purified from porcine stomach; Sigma-Aldrich) 4 g of sucrose (Sigma-Aldrich), 0.35 g of NaCl (Sigma-Aldrich), 0.2 g of $CaCl_2$ (Sigma-Aldrich) and 0.2 g of KCl (Sigma-Aldrich). The pH was adjusted to 6.8 using NaOH.

2.3. Strains, growth conditions and biofilm formation

Two strains from American Type Culture Collection (ATCC) were used: *S. mutans* ATCC 25175 and *C. albicans* ATCC 10231. The media used to seed the cultures of *C. albicans* and *S. mutans* were Sabouraud Dextrose agar (SDA, Difco, Le Pont de Claix, France) and Brain Heart Infusion agar (BHI Agar; Difco), respectively. Colonies of *S. mutans* were suspended in 10 mL of BHI broth (Difco) and incubated for 24 hours in 5% CO_2 at 37 ºC. In turn, *C. albicans* colonies previously cultivated on SDA were suspended in 10 mL of Sabouraud Dextrose broth (Difco) and incubated overnight at 37 °C (120 rpm). After, bacterial and fungal cells were recovered by centrifugation (6,500 × *g* for 5 minutes at 15 °C) and the cell pellets, washed twice with 10 mL of saline solution at 0.85%. The number of fungal cells was adjusted to a concentration of 10^7 cells/mL in artificial saliva, using a Neubauer counting chamber, while bacterial cells were spectrophotometrically adjusted to 10^8 cells/mL. Mixed biofilms were formed in wells of 6-well plates (Costar, Tewksbury, USA). For this, 4 mL of microbial

suspension (1×10^7 cells/mL *C. albicans* + 1×10^8 cells/mL *S. mutans*) were added in the wells, and plates were incubated at 5% CO_2 at 37 °C, during 96 hours. Artificial saliva was renewed every 24 hours.

2.4. Exposure to sucrose and pH measurement

After the biofilm formation period, artificial saliva was completely removed by gentle aspiration, and 2 mL of sucrose were pipetted in the wells containing the resulting biofilms. Sucrose solutions were prepared at 10, 20 or 30%, and remained in contact with biofilms for 1, 3 or 5 minutes. After each of these periods, the sucrose solution was completely removed and the pH was determined immediately (t0), and at 1 (t1), 3 (t3), 5 (t5) and 10 (t10) minutes after sucrose removal. For the measurements, biofilms were scraped from the wells using a cell scraper (Kasvi), transferred to microtubes (MCT-200-C-Axygen) with the aid of a pipette, and a micro pH electrode (PHR-146 Micro Combination pH Electrode - Fisher Scientific) previously calibrated with standards (pH 7.0 and 4.0) was placed in contact with biofilms. Biofilm pH before exposure to sucrose was also measured (Baseline). All tests were performed in triplicate, on three separate occasions.

2.5. Statistical analysis

The normality of the data was verified by Shapiro-Wilk's test, using the statistical program SigmaPlot version 12.0 (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). Data were analyzed by 3-way analysis of variance, followed by Fisher LSD's test. All tests were performed with a significance level of 5%.

3. Results

A marked decrease was observed in pH values measured 1 minute after removal of the sucrose solutions (t1) when compared to the other times, being significantly different from the baseline values ($p < 0.033$), regardless of the contact time with biofilms or the sucrose concentration (Fig. 1). Overall, an increasing trend in pH values from t3 was observed, especially for the shortest contact times of the biofilm with the sucrose solutions (1 and 3 minutes).

Significant differences in pH values were observed for biofilms treated with 10 and 30% sucrose solutions ($p < 0.025$), but not between 10 and 20% ($p = 0.460$) or 20 and 30% ($p = 0.065$). For the 10% sucrose solution (Fig. 1A), pH values observed for 1- and

3-minute exposure times were not significantly different from each other ($p = 0.406$), and were significantly higher when compared to 5 minutes ($p < 0.028$). At this concentration, t1, t3, t5 and t10 did not differ from each other ($p > 0.104$). For sucrose at 20% (Fig. 1B), despite no significant differences were observed among the times of exposure to sucrose ($p > 0.212$), values observed for t1 were significantly different from those seen for t3, t5 and t10 ($p < 0.008$). For sucrose at 30% (Fig. 1C), exposure times of 3 and 5 minutes did not produce significant differences in the biofilm pH ($p = 0.199$), but were significantly different from t1 ($p < 0.028$). Only for sucrose at 30% the pH analyzed immediately after sucrose removal (t0) significantly differed from the baseline ($p < 0.047$).

The critical pH of hydroxyapatite dissolution (5.5) was attained in biofilms exposed during 3 minutes to sucrose at 20% (pH measured 1 minute after sucrose removal; Fig. 1B), as well as in those exposed during 1 minute to sucrose at 30% (pH determined immediately after sucrose removal; Fig. 1C).

4. Discussion

Changes in biofilm pH are paramount for the development of caries lesions (Islam et al., 2007), so that *in vitro* models that are able to reproduce the *in vivo* changes in biofilm pH may aid researchers in investigating such changes, as well as possible factors that might affect this pattern. The present study showed that biofilms of *S. mutans* and *C. albicans* exposed to 20% sucrose solution for 3 minutes (Fig. 1B) exhibited pH drop and recovery similar to that described *in vivo*, despite at a much higher speed.

Sucrose is metabolized by *S. mutans*, leading to a decrease in the pH of the medium caused by the production of lactic acid (Marsh and Martin, 2009). Biofilms exposed to 30% sucrose had significantly lower pH values when compared to those exposed to sucrose at 10% (Fig. 1). In fact, such trend was somehow expected, given that a greater sugar availability would lead to a higher degree of fermentation, thus decreasing the biofilm pH. Interestingly, pH values related to longer exposure times (3 and 5 min) to 30% sucrose were not as low as that observed for the shortest exposure time (1 min). Although the present protocol does not provide data to explain the above-mentioned trend, it is possible that the longest exposure times might have favored a higher degree of neutralization of the acids by *Candida albicans*. In fact, a previous study reported

that this microorganism has the ability to metabolize lactic acid as a carbon source, and thus rapidly neutralizes acidic environments (Danhof et al. 2016).

An interesting aspect of the model was the possibility to observe the four phases described by Bowen (2013) regarding the pH changes in dental biofilm under *in vivo* conditions. Thus, exposure of the mixed biofilm to 20% sucrose during 3 minutes allowed the observation of (1) the baseline pH prior to exposure to sugar; (2) the initial pH drop after exposure to sucrose; (3) the time at which the critical pH for hydroxyapatite dissolution was reached; and (4) the recovery phase. Nonetheless, one major difference between the present *in vitro* data in relation to *in vivo* conditions (Stephan, 1944) is the time that each phase lasted. Despite an initial pH drop occurred after sucrose exposure both *in vivo* and *in vitro*, the fall and permanence of biofilm pH to values below 5.5 were faster *in vitro* compared to *in vivo* conditions. Furthermore, the *in vivo* recovery phase was shown to take longer than the 3 minutes observed *in vitro*. These differences in phase time intervals, almost 10-fold faster *in vitro*, may be related to intrinsic features of the *in vitro* model used, including biofilm thickness, surface for biofilm growth (polystyrene plates), lack of salivary flow and limited microbiota.

The high mucin levels might also have played an important role in pH recovery, since this protein is likely to be the main buffer present in artificial saliva (Cheaib and Lussi, 2013). This protein is also found in natural saliva, but at lower concentrations (0.0027 g/L) than that used in the present study (1 g/L). Such high mucin concentrations were used in order to compensate the lack of continuous nutrient renewal in the *in vitro* model. Although the high mucin levels may have influenced the pH recovery time, it is worth mentioning that the biofilm had no further contact with saliva after exposure to sucrose, so that any buffering effect of mucin would be related to its residual levels in the biofilm.

The *in vitro* biofilm was probably thinner and more porous compared to that naturally developed on tooth surfaces, which tends to facilitate the diffusion of sugars and buffers. Regarding the composition of these biofilms, a greater variety of microorganisms is observed *in vivo*, and there may be other microorganisms that also contribute to acid production, thus affecting the resulting pH and the time for neutralization. The use of biofilm microcosm models could partially overcome the limitations of the current protocol, while keeping the advantages of the well-controlled *in vitro* conditions.

To conclude, sucrose concentration and exposure time are important variables affecting the biofilm pH. In this sense, the use of a 20% sucrose solution in a dual-species biofilm of *S. mutans* and *C. albicans* during three minutes allowed the observation of pH changes resembling those seen in the dental biofilm *in vivo*, despite at a higher speed when compared to *in vivo* conditions. This experimental model may assist in preclinical research assessing changes in biofilm pH and the effects of therapeutic agents on this important variable.

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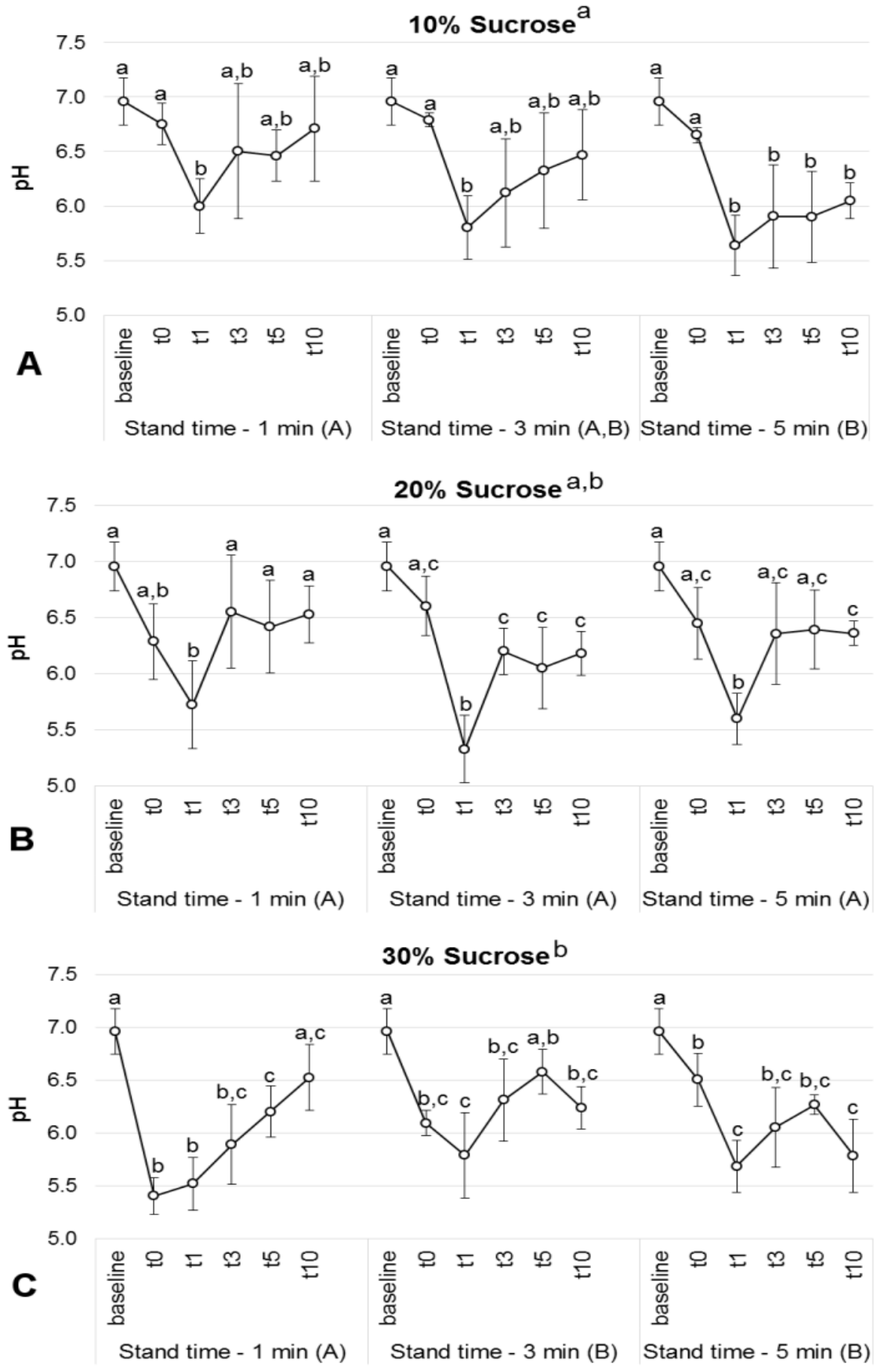
Figure Caption

Fig. 1. Mean pH values determined after exposure of the mixed biofilm of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175 to 10% sucrose (A), 20% sucrose (B) and 30% sucrose (C), as a function of the exposure time to sucrose and time of pH determination. Baseline: pH values determined before biofilm exposure to sucrose; t0: pH determined immediately after sucrose removal; t1, t3, t5 and t10: pH determined at 1, 3, 5 and 10 minutes after sucrose removal, respectively. Different capital letters between parentheses denote significant differences among exposure times to sucrose within the same sucrose concentration (Stand time). Different lowercase letters represent significant differences among pH values at each individual graph, as well as among sucrose concentrations. Vertical bars represent the standard deviation of the means (Student-Newman-Keuls, $p < 0.05$, $n = 3$).

Figure 1


10% Sucrose a
20% Sucrose a,b
30% Sucrose b
pH
7.5
7.0
6.5
6.0
5.5
5.0
A
B
C
baseline
t0
t1
t3
t5
t10
Stand time - 1 min (A)
Stand time - 3 min (A,B)
Stand time - 5 min (B)
Stand time - 3 min (A)
Stand time - 5 min (A)
Stand time - 1 min (A)
Stand time - 3 min (B)
Stand time - 5 min (B)

Capítulo 3

Sodium hexametaphosphate and fluoride affect F, Ca, Pi and pH of dual-species biofilms after sucrose exposure

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Sodium hexametaphosphate and fluoride affect F, Ca, Pi and pH of dual-species biofilms after sucrose exposure

Abstract

Objectives: To evaluate the effects of sodium hexametaphosphate (HMP), associated or not to fluoride (F), on the concentrations of F, calcium (Ca) and phosphorus (Pi), and pH of mixed biofilms of *Streptococcus mutans* and *Candida albicans*, before and after exposure to sucrose.

Methods: Biofilms were treated three times (72, 78 and 96 hours after the beginning of their formation) at three HMP concentrations (0.25, 0.5 or 1%), associated or not with F (500 ppm). Solutions containing 500 and 1100 ppm F, and artificial saliva were also tested as controls. Exposure of biofilms to 20% sucrose solution occurred after the third treatment (96 h). Biofilm pH was measured (micro electrode) and the concentrations of F (ion-specific electrode), Ca (Arsenazo III), Pi (colorimetric method), and HMP (heating in acid medium) were determined in the solid and fluid phases of the biofilm. Data were submitted to 2-way ANOVA, followed by Fisher's LSD test ($p < 0.05$).

Results: The association of HMP/F significantly increased F and decreased Pi levels in the fluid compared to 500 ppm F. On the other hand, HMP decreased Ca in the fluid and HMP in the biomass. Exposure to sucrose significantly decreased all ions in the fluid and biomass, except for HMP in the biomass. Treatment with 1% HMP and F resulted in the highest pH values in the biofilm after exposure to sucrose.

Conclusions: HMP significantly affects the biofilm, increasing F and Pi concentrations in the fluid, and keeping the pH close to neutral values, even after sucrose exposure.

Clinical significance: The buffering effect promoted by the association of fluoride and HMP on the biofilm pH, along with the effects on fluoride and phosphorous concentrations in the fluid, provided new insights into the mechanisms of action of formulations containing both compounds.

1. Introduction

Dental caries is a multifactorial and sugar biofilm-dependent disease [1], caused by acid-producing bacteria due to the consumption of fermentable carbohydrates, what progressively results in enamel demineralization [2]. *Streptococcus mutans* consists of one of the main etiological agent of dental caries due to its ability to colonize dental surfaces, metabolize carbohydrates, produce lactic acid, and grow in acid medium [3, 4].

Although *S. mutans* is considered the major pathogenic agent related to dental caries, this microorganism does not act by itself. *Candida albicans* has been often associated to the pathogenic biofilm, especially in early childhood caries [5]. *C. albicans* is also the fungus most frequently found in human mucosa, usually associated to polymicrobial biofilm formation on soft tissues and acrylic surfaces.

The decline of dental caries prevalence has been associated to the use of fluoride (F) products, and dentifrices contribute expressively to these trends [6]. Concomitantly, an increased prevalence of dental fluorosis has been be observed, being the ingestion of F from dentifrices by children younger than 6 years of age considered as the main reason for this pattern [7, 8]. Although the use of low-fluoride dentifrices is considered an alternative for minimizing F intake from toothbrushing, evidence regarding their clinical effectiveness is scarce, with reports indicating a lower clinical performance in caries-active children [9]. Considering the scenario above, the supplementation of low-fluoride toothpastes with calcium and/or phosphate salts has been studied as an alternative to increase the effectiveness of these products.

Sodium hexametaphosphate (HMP) is a phosphate salt widely used in industry as an antimicrobial agent due to its ability to increase the permeability of the bacterial wall [10], and to disperse the microbial biofilm [11]. *In vitro* studies have demonstrated that low-fluoride dentifrices (250 µg F/g) supplemented with HMP present similar effect of conventional dentifrices (1100 µg F/g) on enamel demineralization [12]. However, the mechanism by which HMP acts on the dental biofilm remains uncertain [13, 14].

Considering the aforementioned information, the present study aimed to assess the effect of HMP, associated or not to F, on the concentrations of F, calcium (Ca), phosphorus (Pi), and on the pH of mixed biofilms of *S. mutans* and *C. albicans*, before and after exposure to sucrose. The null hypotheses were that HMP would not be

adsorbed in the biofilm, nor interfere in F, Ca, Pi and HMP concentrations in the biofilm, performed *in vitro*.

2. Materials and methods

2.1. Microorganisms and growth conditions

The reference strains used from the American Type Culture Collection (ATCC) *C. albicans* (ATCC 10231) and *Streptococcus mutans* (ATCC 25175) which were kept at -70° C in glycerin. Cultures of *S. mutans* were seeded in Brain Heart Infusion (BHI Agar; Difco) and *C. albicans* in Sabouraud Dextrose Agar (SDA, Difco, Le Pont de Claix, France). *S. mutans* plates were maintained in 5% CO_2 at 37° C for 24 hours, and plates of *C. albicans* were incubated for 24 hours at 37° C. After growth in agar, colonies of *S. mutans* was suspended in 10 mL of BHI broth (Difco) and incubated statically overnight in 5% CO_2 at 37° C. *C. albicans* was suspended in 10 mL of Sabouraud Dextrose broth (Difco) and incubated at 37° C overnight under shaking at 120 rpm [15]. The cells were then recovered by centrifugation (8000 rpm, 5 min) and washed twice with 10 mL of saline (0.85% NaCl). Using a Neubauer Chamber, the fungal cells were adjusted to a concentration of 10^7 cells/mL in artificial saliva [16]. The amount of bacterial cells were adjusted spectrophotometrically (640 nm) at a concentration of 10^8 cells/mL of saline (0.85% NaCl) [16]. The artificial saliva used [17] was supplemented with sucrose and thus had the following composition for 1 L of deionized water: 2 g of yeast extract (Sigma-Aldrich, St Louis, USA), 5 g of bacteriological peptone (Sigma-Aldrich), 4 g of sucrose (Sigma-Aldrich), 1 g of mucin (Sigma-Aldrich), 0.35 g NaCl (Sigma-Aldrich), 0.2 g $CaCl_2$ (Sigma-Aldrich) and 0.2 g KCl (Sigma-Aldrich). The pH of the saliva was adjusted with NaOH to 6.8. To form the biofilms, 4 mL suspension (1×10^7 cells/mL *C. albicans* + 1×10^8 cells/mL *S. mutans*) in artificial saliva were added to the wells of 6-well microtiter plates (Costar - Coring, USA). These were incubated at 37° C for 72 hours and every 24 hours 2 mL of the supplement with sucrose artificial saliva was renewed.

2.2. Treatment of biofilms and pH measurement

The biofilms were treated 3 times (72, 78 and 96 hours after the start of their formation) for 1 minute [18] with solutions of : 0.25% (0.25HMP), 0.5% (0.5HMP), 1%

(1HMP), 0.25HMP + 500 ppm F (0.25 HMP/F), 0.5 HMP + 500 ppm F (0.5 HMP/F) and 1HMP + 500 ppm F (1 HMP/F). As controls, solutions containing 500 and 1100 ppm F, and artificial saliva without F and HMP were used. The biofilms were gently washed with 1 mL of artificial saliva for 10 seconds after the third treatment. Following, biofilms were scraped with a cell scraper and transferred, with the assist of a pipette, to microtubes, for pH determination using a pH electrode (PHR-146 Micro Combination pH Electrode, Fisher Scientific, California-USA), previously calibrated with standards with pH 7.0 and 4.0. The experiments were performed in triplicate, on three different occasions.

In another set of experiments, after the third treatment artificial saliva was removed and the biofilms were exposed to a 20% sucrose solution for 3 minutes, as a cariogenic challenge [19]. The sucrose solution was then removed, biofilms were scraped and transferred to microtubes (within 1 min after removal of the sucrose solution) allowing pH determination, exactly as described above [19].

2.3. Analysis of F, Ca and Pi in the biofilm fluid

The microtubes containing the scraped biofilms were centrifuged (1,5267 × *g*) at 4º C for 5 minutes, and the biofilm fluid was collected [20]. F was analyzed using a specific electrode (Orion 9409 BN) and reference electrode (Orion 900100), both coupled to a potentiometer (Orion, Thermo Scientific, Beverly-USA). The calibration curves for F analysis in the fluid were performed using 0.09, 0.18, 0.36, 0.72, and 1.44 μg F/mL standards (for biofilms treated with F-free solutions) and 6.25, 12.5, 25, 50 and 100 μg F/mL (for biofilms treated with solutions containing F). A total ionic strength adjustor buffer (TISAB II) was used under the same conditions as the samples, at a 1:1 ratio.

Calcium was measured by spectrophotometry on a plate reader (EONC Spectrophotometer, Biotek, USA) at wave length of 650 nm, adapting the method described by Vogel [21]. In brief, Arsenazo III was used as a colorimetric reagent. An aliquot of 5 μL in duplicate for both standards and samples was mixed with 50 μL of Arsenazo III and 50 μL of deionized water. They were then agitated for 60 seconds in the microplate reader, promoting the reaction between the sample and Arsenazo III before obtaining the resulting absorbances.

Total phosphorus was measured according to the method of Fiske and Subbarow [22]. The determination of P from HMP was performed using the protocol proposed by Anderson *et al.* [23]. For the samples that were exposed to sucrose, the determination of P from HMP, the boiling-water bath process was replaced by storage of the solutions at 60° C for 6 hours.

2.4. Analysis of F, Ca and Pi in the biofilm biomass

For the analysis of the inorganic composition of the biofilm biomass, 0.5 mol/L HCl was added to the microtubes containing the biofilms at the proportion of 0.5 mL/10.0 mg plaque wet weight [24], and homogenised. The resulting mixture was kept for 3 hours at room temperature and under constant stirring (120 rpm), and then centrifuged (11,000 × *g*) for 1 minute [25]. A known amount of the liquid was removed and the same volume of 0.5 mol/L NaOH was added.

Fluoride was analyzed as previously described, using standards containing 0.09, 0.18, 0.36, 0.72, and 1.44 μg F/mL (for biofilms treated with F-free solutions) and 0.8, 1.6, 3.2, 6.4 and 12.8 μg F/mL (for biofilms treated with solutions containing F). For biofilms exposed to sucrose, the calibration curve was performed using 0.2, 0.4, 0.8, 1.6 and 3.2 μg F/mL standards. Ca and P was determined as described for the biofilm fluid.

2.5. Determination of HMP and Ca after cell lysis

To quantify the HMP and Ca levels bound to the microorganism, was added 50 uL of HCl 1 mol/L in microtubes containing 10 ug of biomass, being homogenized. Then, the microtubes were placed in boiling water (100 °C, 30 minutes) to promote cell lysis [26] and HMP hydrolysis [27] and centrifuged (11.000 × *g*, 1 minute) [25]. An known amount of the liquid was removed and the same volume of NaOH 1 mol/L was added to Ca [21] and HMP analysis [22].

2.6. Determination 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-}, CaF^+ and HF^0) were calculated from the concentrations (mmol/L) of calcium, fluoride, and phosphorus in the biofilm fluid of each group [28]. Also, degree of saturation (DS) of the solid phases of hydroxyapatite (HA) and calcium fluoride (CaF_2) were determined. All calculations were performed for conditions at 37° C, pH and

density of 1.0 g/cm^3 by the PHREEQC Interactivef (version 2.18.3, U.S. Geological Survey Branch of Information Services, Denver, CO, USA) speciation program. The pH values determined at the end of the experiments for each group were used for the calculation of IA and DS.

2.7. Statistical analysis

Data were analyzed by using the statistical program SigmaPlot version 12.0 (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 was performed, followed by Fisher LSD's test. All tests were performed with a significance level of 5%.

3. Results

3.1. F, Ca and Pi in the biofilm fluid

The concentrations of F, Ca and Pi ions and HMP in the biofilm fluid significantly decreased after exposure to sucrose ($p < 0.001$), regardless of the treatment (Figure 1A). For F concentrations, a direct dose-response relationship was observed between F concentrations in the treatment solutions and the resulting levels in the biofilm ($p < 0.001$; Figure 1A). The association HMP/F led to higher fluoride levels when compared to 500 ppm F, but lower than the 1100 ppm F for biofilms not exposed to sucrose ($p < 0.001$). Regarding Ca levels, the groups treated with 1100 ppm F, and 0.5 and 1HMP associated or not with F, did not show detectable levels of Ca in the biofilm liquid, regardless of the exposure to sucrose (Figure 1B). Furthermore, Ca was not detected in biofilms treated with 0.25HMP, both with and without F (Figure 1B), after exposure to sucrose. Pi concentrations were higher in groups treated with HMP without F, both before and after exposure to sucrose ($p < 0.001$; Figure 1C). All HMP and HMP/F groups presented a dose-response relationship between HMP levels in the treatment and P concentrations in the fluid, with and without sucrose exposure, except for 0.5 and 1% groups after sucrose exposure ($p < 0.001$; Figure 1C). Similar trends were also observed regarding HMP concentrations, which presented dose-response relationship according to the treatment solutions ($p < 0.001$; Figure 1D).

3.2. F, Ca and Pi in the biofilm biomass

A direct dose-response relationship was observed between F concentrations in the treatment solutions and the resulting F levels in the biomass. Groups treated with 0.25 and 0.5HMP and F presented F values significantly lower in comparison to 500 ppm F ($p < 0.001$) regardless of the exposure to sucrose, while values observed for the 1% HMP/F group (both with or without sucrose exposure, $p < 0.091$; Figure 2A) were similar to the 500 ppm F group. Regarding Ca concentrations, treatment with HMP or HMP/F did not affect the resulting values, under both conditions of sucrose exposure. In addition, Ca was not detected in all groups treated with HMP, associated or not to F, after sucrose exposure ($p > 0.069$; Figure 2B). Furthermore, biofilms treated with 1100 ppm F presented higher Ca concentrations in comparison to the other groups, exposed or not to sucrose ($p < 0.001$; Figure 2B). As for Pi levels (Figure 2C), the higher HMP concentration in treatment solution, the higher Pi values in biomass before sucrose exposure, but such dose-response relationship was not observed after sucrose exposure. Groups treated with 0.25HMP and 0.5HMP presented higher Pi concentrations compared to their HMP/F counterparts without sucrose exposure. After sucrose exposure, only groups treated with 500 and 1100 ppm F presented similar Pi concentrations compared to those not exposed to sucrose. Prior to sucrose exposure, HMP was not detected in biofilms of any HMP groups. However, after sucrose exposure, a dose-response relationship regarding HMP levels in the biomass and those in the treatment solutions was observed (Figure 2D), despite no significant differences were observed between HMP groups with or without F.

After cells' lysis, higher Ca concentrations were observed in the groups treated with 0.5 and 1HMP, with and without F, before sucrose exposure (Figure 3A). Furthermore, all HMP groups were not significantly different compared to control after sucrose exposure. Regarding HMP concentrations, all HMP groups presented higher HMP values compared to control before sucrose exposure, besides presenting a dose-response relationship according to their concentrations. The groups 0.5HMP, 1HMP and 1HMP/Fexposed to sucrose presented significantly difference from the negative control (Figure 3B).

3.3. Biofilm pH

The pH of all biofilms significantly decreased after exposure to sucrose ($p < 0,001$). A dose-response relationship was observed between F levels in the treatment solutions and the pH of the biofilms, both before and after sucrose exposure. Treatments with HMP/F led to higher pH values in comparison to all the other groups not exposed to sucrose (Table 1). Furthermore, the pH of biofilms treated with 0.25 and 0.5% associated to F was not significantly different from that observed for the 1100 ppm F group after exposure to sucrose. The association of 1HMP with F led to the highest pH value in comparison to all the other groups after sucrose exposure.

3.4. Determination of ionic activities and degree of saturation in the biofilm fluid

The degree of saturation in relation to hydroxyapatite was significantly lower in the groups exposed to sucrose in comparison to those not exposed (Table 2). The lowest saturation was observed for the 0,25HMP group. As for the saturation in relation to CaF_2 (Table 2), the highest values were found for groups treated with HMP and F, while the lowest values were observed for groups treated with only HMP. The 1100 ppm F presented a lower degree of saturation (HA and CaF_2) than 500 ppm F.

The estimated formation of $CaHPO_4^0$ (Figure 4A e B) was higher in the groups treated with 0.25HMP and 0.25HMP/F, without significantly difference between these groups. Regarding the formation of HPO_4^{2-} (Figure 4B), a dose-response relationship was observed between the concentrations of 0.5 and 1HMP. The group treated with 1100 ppm F reduced $CaHPO_4^0$ formation and increased HPO_4^{2-} formation in comparison to the group 500 ppm F. The highest CaF^+ formation was observed for the group treated with 500 ppm F. The association of F with 0.25HMP provided higher CaF^+ formation in comparison to its counterpart without F (Figure 4C). The possible formation of HF^0 (Figure 4D) was higher in the groups associated with F, both prior and after sucrose exposure.

4. Discussion

The present study aimed to assess the influence of HMP and F, alone or in association, on the inorganic composition, and on the pH of a dual-species biofilm of *S. mutans* and *C. albicans in vitro* before and after exposure to sucrose. Treatments with

HMP and F led to significant increases in F concentrations in the biofilm fluid before exposure to sucrose, while treatments with HMP alone significantly increased Pi levels both in the solid and fluid phases of the biofilms. The pH was also influenced by treatment with HMP and F, being higher when the biofilm received HMP and F in association. However, HMP reduced Ca concentrations in the biofilm (biomass and fluid phase). Therefore, the null hypotheses of this study were rejected.

The pH fall in the biofilms studied is mainly due to the production of lactic acid by *S. mutans* after sucrose exposure, which is responsible for the progression of caries lesions under clinical conditions. In the present study, treatment with HMP solutions (associated or not to F) influenced pH drop, with higher HMP concentrations associated to F leading to higher pH values, both prior and after sucrose exposure, in line with studies reported in literature [29, 30]. Regarding the influence of HMP alone, the effects on biofilm (solid and fluid phases) seems to be related to the buffering capacity of this inorganic cyclophosphate, what was observed especially at the highest concentration tested [31]. As for the effects from the association between HMP and F, it is known that F is associated with the decrease of *S. mutans* acidogenicity, as well as the inhibition of extracellular polysaccharides synthesis, reducing gene expression associated to glycosyltransferases and glycolysis, events that lead to pH drop [32]. In this sense, samples treated with 1100 ppm F led to a higher availability of F (compared with 500 ppm F), what may have contributed in the maintenance of more neutral pH considering the above-mentioned mechanisms. Furthermore, the maintenance of the pH closer to neutral values in this group (1100 ppm F) and 1HMP (both prior and after sucrose exposure) may be associated to HPO_4^{2-} formation (Figure 3B), which also has a buffering effect [33] (Table 1).

HMP is a cyclic phosphate that strongly binds with metallic ions [34]. In the oral cavity, it has the ability to retain charged ions and ionic species (such as Ca^{2+} and CaF^{+}) by replacing Na^{+} in its cyclic structure, leading to a reticular formation in which Ca^{2+} bridges molecules of HMP [12]. In this sense, Ca^{++} available in the medium (from saliva) binds to HMP, plummeting Ca^{++} concentrations in the biofilm fluid, consequently influencing its saturation regarding HA e CaF_2. A similar pattern was observed for the group treated with 1100 ppm F, what may be justified by its retention in the biofilm biomass by calcium bridging [35]. Lower levels of Ca^{++} also justify the lower saturation in the biofilm fluid regarding CaF_2 and the lower possibility of

$CaHPO_4^0$ and CaF^+ formation. Furthermore, treatment with HMP-containing solutions (with or without F) and 1100 ppm F provided higher Ca^{++} concentrations in the biomass prior to sucrose exposure, which may be associated to metal chelating (characteristic of HMP) [34] and to the Ca^{++} ability to binding to F. It has been shown that F retention in the dental biofilm is mediated by Ca^{++} in the form of precipitated minerals (CaF_2), or bound to the bacterial surface and proteins from the biofilm matrix [35].

Biofilms treated with HMP-containing solutions (associated or not with F) led to lower degrees of saturation in relation to HA and CaF_2 in comparison to the negative control and 500 ppm F, negatively affecting the dynamics of demineralization and enamel remineralization [36]. These results disagree with previous studies which showed that HMP, alone or in association with F, promotes a high degree of saturation in relation to HA and CaF_2 [13, 37]. It is noteworthy, however, that the biofilms in the present study were not formed on a mineralized substrate (*e.g.*, enamel, dentine or hydroxyapatite specimens) and did not mimic the salivary flow that occurs intraorally, both of which could provide conditions for ionic exchange (and consequently a source of Ca^{++}), what thus influencing the resulting degree of saturation. Despite the lower degree of saturation discussed above, it was possible to observe that the higher the HMP concentration in the treatment solutions, the higher the HMP availability in the biofilm (solid and fluid phases), thus increasing its availability for adsorption on enamel under clinical conditions [38], leading to the formation of a barrier on the enamel surface (as discussed above), acting against the mineral loss under cariogenic challenge [12, 13, 14].

The ability of HMP to bind metallic ions may be related to increases in P levels in the biofilm, mainly at higher concentrations without F association. Studies reported that yeasts are able to develop mechanisms for nutrients adsorption under deprivation conditions, as in the case of Mg^+ sequestration [39], which is supposedly bound to HMP. Based on the above, these microorganisms may perform endocytosis and capture nutrients bound to the HMP molecule, later destroying the molecule in the cytoplasm, subsequently resulting in ion efflux back to the culture medium [39]. Thus, it is possible that HMP from the treatment solution promoted metal chelation in the culture middle, forming HMP-metal complexes that may have been captured by *C. albicans*, which were subsequently metabolized and released back to the culture medium, what could explain the increased P levels and decreased HMP levels in the biofilm fluid observed. Another aspect that deserves comment is the antimicrobial activity of HMP, due to its

ability to increase the permeability of bacteria's outer membrane and glucose transportation when it is bound to Mg^{+} present in the cell wall. This binding justifies the absence of HMP in the extracellular matrix, as presented in the results (Figures 2D and 4A). After sucrose exposure, the acid production would promote the release of the phosphate from the microorganism's wall, releasing it to the extracellular matrix, as observed in the present study. It is also notable that HMP concentrations of 0.5 and 1%, with or without F, promoted higher HMP and Ca levels bound to the microorganisms, which are also bound to HMP molecule (Figure 3A, 3B). This pattern was not observed for the 0.25HMP group, associated or not to F, given that Ca^{++} was detected in the biofilm fluid prior to sucrose exposure in this group.

The availability of Pi in the biofilm fluid was directly related to HMP concentrations (at 0.5 and 1%) in the treatment solutions, suggesting that this phosphate undergoes hydrolysis over time. This is relevant data, since Ca and P levels in the biofilm fluid directly influence the formation of $CaHPO_4^0$ (Figura 3A). It is believed that HMP associated to F releases CaF^{+} to saliva, which can react with H_2PO_4, forming $CaHPO_4^0$ and HF^0 [28]. The present results corroborate this hypothesis, given that HF^0 is in larger proportion in the biofilm fluid of groups treated with HMP and F. It was reported that $CaHPO_4^0$ neutral species is paramount for enamel remineralization, since its diffusion coefficient into subsurface lesions is much higher in comparison to ionic calcium [28]. However, a limitation of the present study is that the only source of Ca^{++} was the culture medium (saliva), what limits ionic exchange, making it difficult to estimate $CaHPO_4^0$ formation. Nonetheless, the study protocol was intentionally planned in order to provide data on the effects of HMP only in the biofilm, without interferences from other sources, such as hydroxyapatite or dental substrate, which could be included in future investigations.

As observed in previous studies, F, Ca and Pi concentrations in biofilm and biofilm fluid decreased after sucrose exposure [40, 41]. Despite such decreases were also noted for groups treated with HMP-containing solutions, the association of 1HMP and F was shown to be effective in keeping the biofilm pH close to neutral values. Furthermore, this combination led to higher F levels in the biofilm fluid after sucrose exposure, what may further reduce demineralization. Thus, it is possible to conclude that HMP significantly affects the dual-species biofilm of *S. mutans* and *C. albicans* biofilms

developed in the present study, increasing F and Pi concentrations in biofilm fluid, and keeping the pH close to neutral values, even after sucrose exposure.

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Table legends

Table 1. Mean values (standard deviation) of pH of the biofilm after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Table 2. Degree of saturation values (SD) in relation to hydroxyapatite (HA) and calcium fluoride (CaF_2) from biofilm fluid, before (no sucrose) and after (sucrose) contact with sucrose according to the groups. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Figures legends

Figure 1. Mean values of F (A), Ca (B), P (C) and HMP (D) in μg / mL of biofilm fluid, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Figure 2. Mean values of F (A), Ca (B), P (C) and HMP (D) in μg / mL of biofilm biomass, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Figure 3. Mean values of Ca (A), HMP (B), in μg / mL after cell lysis, before and after contact with sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Figure 4. Possible formations of $CaHPO_4^0$ (A), HPO_4^{-2} (B), CaF^+ (C), HF^0 (D) in the biofilm fluid before and after contact with the sucrose. Lower case letters present statistical difference between groups ($p < 0.05$). Capital letters present statistical difference between groups prior and after sucrose exposure.

Table 1

	Groups								
	Control	**500 ppm F**	**1100 ppm F**	**0.25 HMP**	**0.5 HMP**	**1 HMP**	**0.25 HMP/F**	**0.5 HMP/F**	**1 HMP/F**
no-sucrose	$6.06^{a,A}$ (0.07)	$6.52^{b,A}$ (0.22)	$7.01^{c,A}$ (0.16)	$6.46^{bd,A}$ (0.26)	$6.60^{bf,A}$ (0.05)	$6.87^{cg,A}$ (0.23)	$7.22^{e,A}$ (0.08)	$7.27^{e,A}$ (0.06)	$7.28^{e,A}$ (0.10)
Sucrose	$4.77^{a,B}$ (0.02)	$6.20^{b,B}$ (0.32)	$6.49^{c,B}$ (0.31)	$5.52^{d,B}$ (0.25)	$5.73^{e,B}$ (0.14)	$6.51^{c,B}$ (0.10)	$6.52^{c,B}$ (0.12)	$6.58^{c,B}$ (0.18)	$6.99^{f,B}$ (0.13)

Different lower case letters show differences between groups and upper case letters show statistical difference in groups before and after exposure to sucrose.

Table 2.

Groups	*Degree of Saturation*			
	HA		CaF_2	
	no sucrose	Sucrose	no sucrose	Sucrose
Control	$0.80^{a,A}$ (0.12)	$-13.60^{a,B}$ (0.97)	$-3.43^{a,A}$ (0.15)	$-5.26^{a,B}$ (0.17)
500 ppm F	$2.84^{b,A}$ (0.32)	$-9.70^{b,B}$ (0.41)	$2.92^{b,A}$ (0.10)	$0.86^{b,B}$ (0.19)
1100 ppm F	$-25.16^{c,A}$ (0.36)	$-29.97^{c,B}$ (0.87)	$-2.44^{c,A}$ (0.02)	$-3.22^{c,B}$ (0.03)
0.25HMP	$-4.39^{d,A}$ (0.78)	$-37.83^{d,B}$ (0.21)	$-4.44^{d,A}$ (0.18)	$-10.32^{d,B}$ (0.01)
0.5HMP	$-29.18^{e,A}$ (0.22)	$-34.86^{e,B}$ (2.91)	$-9.93^{e,A}$ (0.17)	$-10.11^{d,A}$ (0.31)
1HMP	$-26.83^{f,A}$ (0.15)	$-30.42^{cf,B}$ (0.12)	$-10.01^{ef,A}$ (0.14)	$-10.25^{d,A}$ (0.06)
0.25HMP/F	$-0,60^{g,A}$ (1.74)	$-32.25^{gh,B}$ (0.25)	$2.41^{cg,A}$ (0.22)	$-3.72^{e,B}$ (0.04)
0.5HMP/F	$-31.61^{h,A}$ (0.10)	$-31.61^{fh,A}$ (0.10)	$-3.81^{h,A}$ (0.10)	$-3.81^{e,A}$ (0.10)
1HMP/F	$-27.90^{ef,,A}$ (3.27)	$-29.03^{ci,A}$ (0.06)	$-3.01^{bi,A}$ (0.03)	$-3.60^{e,B}$ (0.07)

Distinct lower cases letters indicate statistical significance among the groups. Distinct upper cases letters indicate statistical difference between no sucrose and sucrose (Fisher LSD's test; p <0.05).

Figure 1

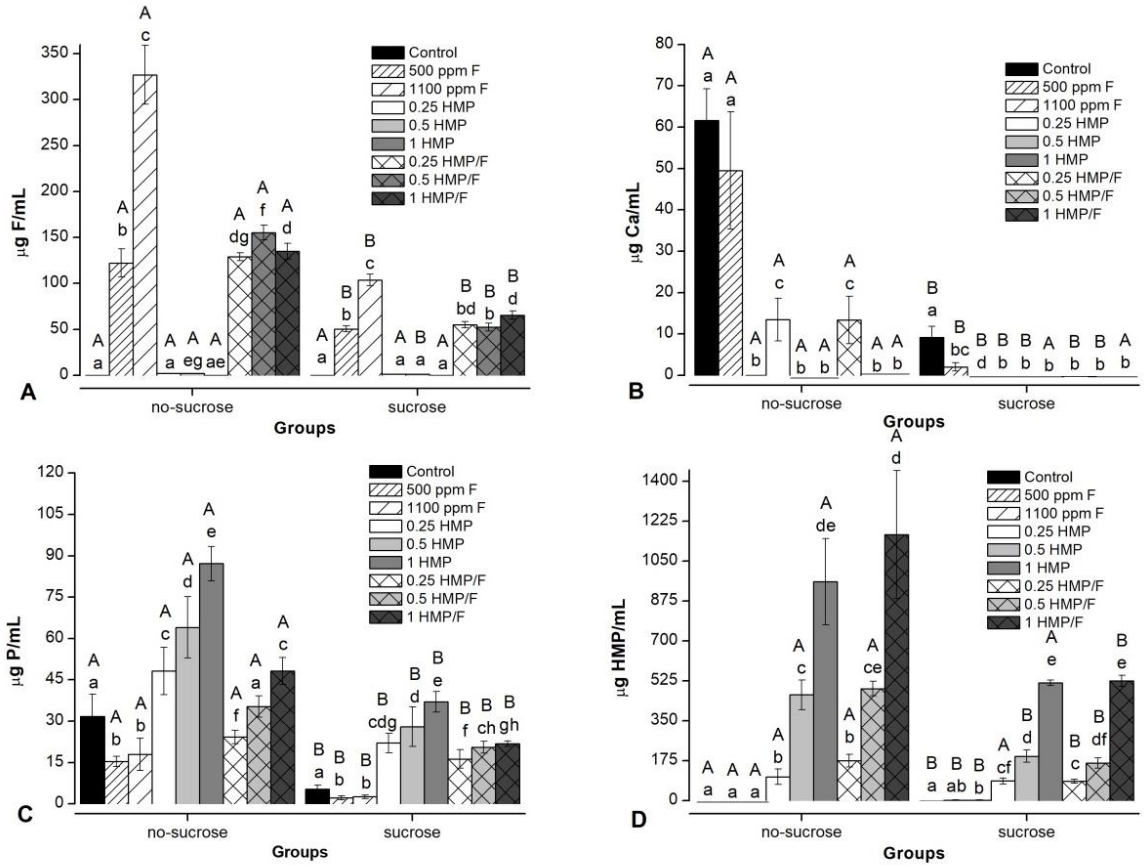

A
µg F/mL
Control
500 ppm F
1100 ppm F
0.25 HMP
0.5 HMP
1 HMP
0.25 HMP/F
0.5 HMP/F
1 HMP/F
no-sucrose
sucrose
Groups
B
µg Ca/mL
C
µg P/mL
D
µg HMP/mL

Figure 2.

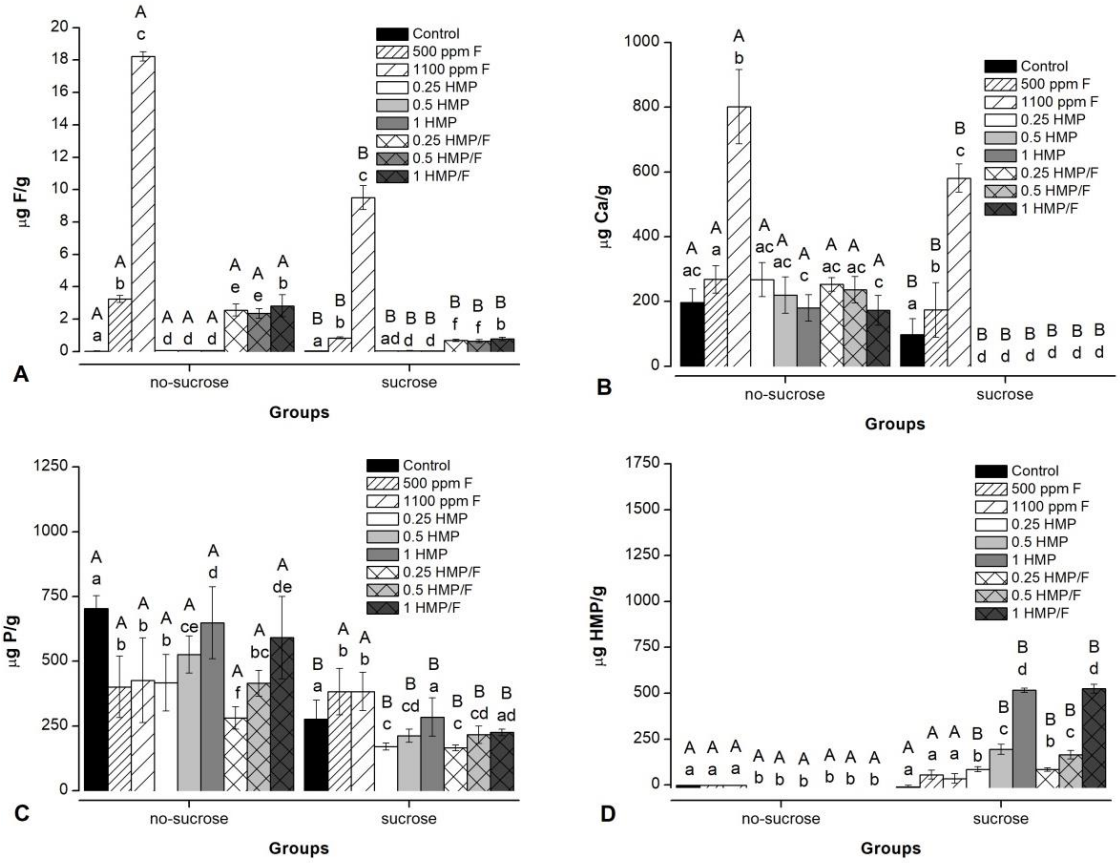

A
µg F/g
Control
500 ppm F
1100 ppm F
0.25 HMP
0.5 HMP
1 HMP
0.25 HMP/F
0.5 HMP/F
1 HMP/F
no-sucrose
sucrose
Groups
B
µg Ca/g
C
µg P/g
D
µg HMP/g

Figure 3.

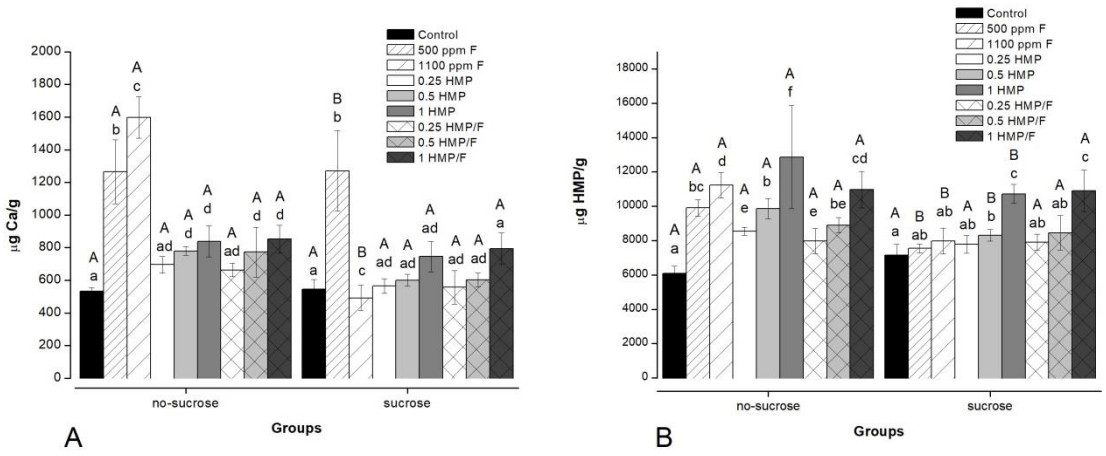
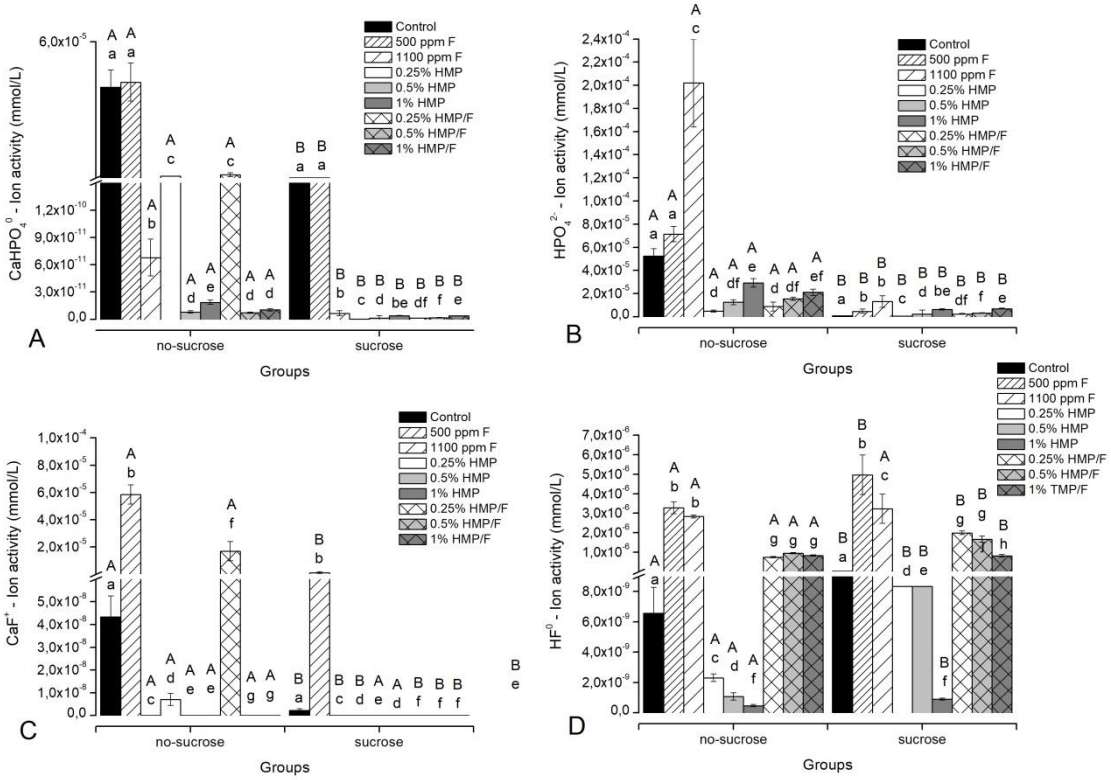

Control
500 ppm F
1100 ppm F
0.25 HMP
0.5 HMP
1 HMP
0.25 HMP/F
0.5 HMP/F
1 HMP/F
µg Ca/g
µg HMP/g
no-sucrose
sucrose
Groups
A
B

Figure 4.


A
B
C
D
Control
500 ppm F
1100 ppm F
0.25% HMP
0.5% HMP
1% HMP
0.25% HMP/F
0.5% HMP/F
1% HMP/F
1% TMP/F
no-sucrose
sucrose
Groups
CaHPO4 0 - Ion activity (mmol/L)
HPO4 2- - Ion activity (mmol/L)
CaF+ - Ion activity (mmol/L)
HF0 - Ion activity (mmol/L)

Anexos

ANEXO A

REFERÊNCIAS INTRODUÇÃO GERAL

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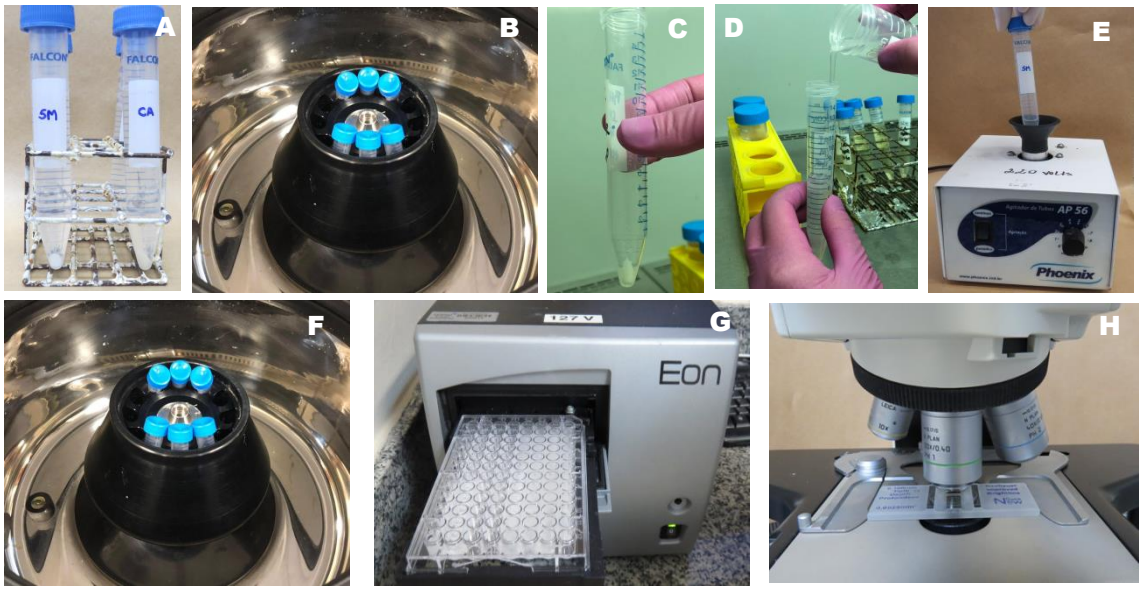
ANEXO B

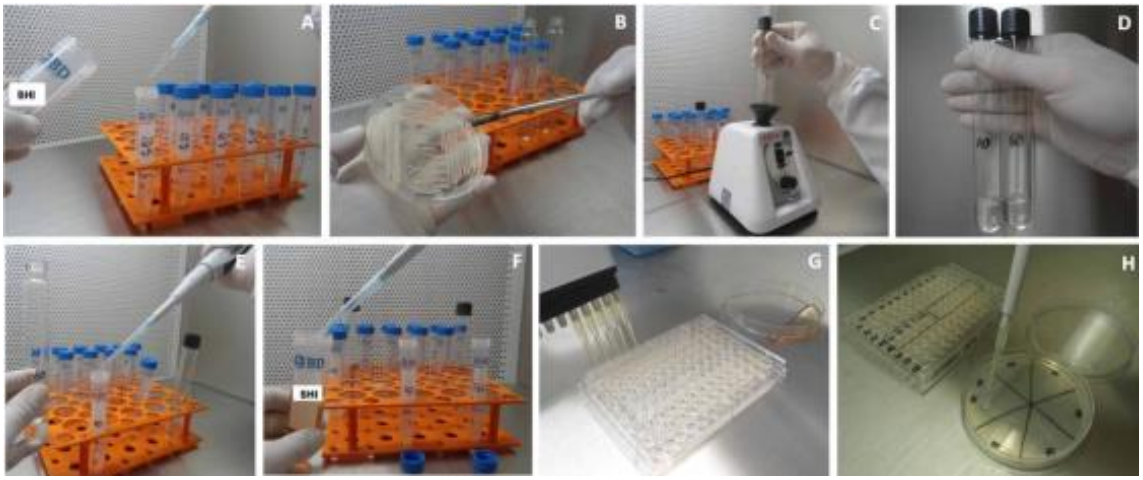
ILUSTRAÇÃO ESQUEMÁTICA DA PREPARAÇÃO DO INÓCULO DA CEPA DE *S. MUTANS* E *C. ALBICANS*



(A) Preparação do pré-inóculo de *S. mutans* ATCC 25175 e *C. albicans* ATCC 10231; (B) Centrifugação do inóculo a 8000 rpm durante 5 min; (C) Obtenção do pellet de células e descarte do sobrenadante; (D) Lavagem das células de *S. mutans e C. albicans* NaCl (0,85%), (E) Homogeneização em vórtex e (F) Centrifugadas novamente (foram realizadas duas lavagens com NaCl); (G) Alíquotas de 200 µl adicionadas aos poços de placas de 96 poços e levadas para leitura em espectrofotômetro a 640 nm afim de ajustar o número de células de *S. mutans* para 10^8 células/mL em saliva artificial (SA); (H) O número de células de *C. albicans* ajustado para 10^7 células/mL em SA usando uma câmara de Neubauer.

ANEXO C

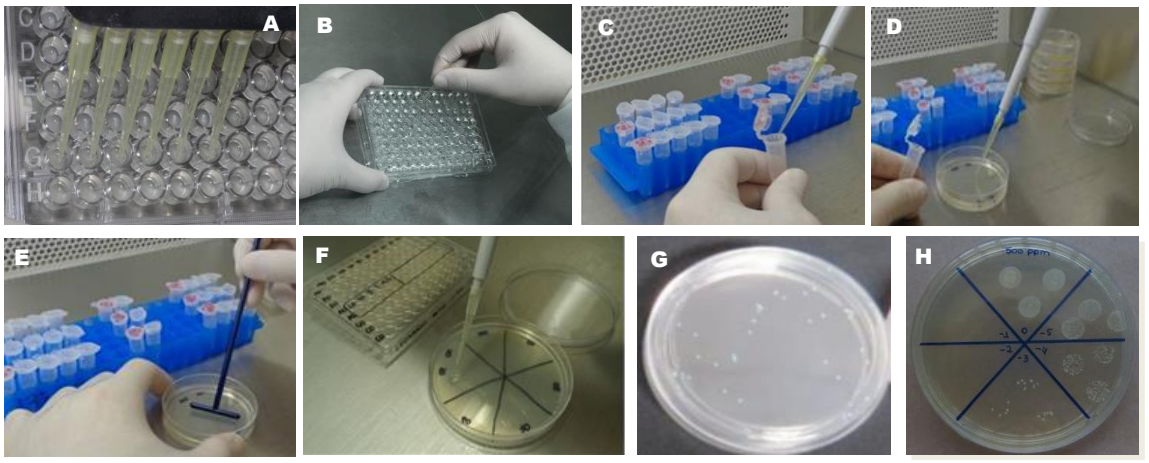
DETERMINAÇÃO DA CONCENTRAÇÃO INIBITÓRIA MÍNIMA



(A) Diluição (1:5) de cada concentração de hexametafosfato de sódio no meio de cultura BHI caldo (*S. mutans*) e RPMI (*C. albicans*); (B) Inoculação de colônias de *S. mutans e C. albicans* em solução salina 0,85%; (C) Homogenização em vórtex da suspensão da cepa; (D) Ajuste da turbidez da suspensão da cepa ao padrão 0,5 da escala McFarland; (E) Diluição (1:5) da suspensão celular em solução salina; (F) Diluição (1:20) da suspensão celular no meio BHI caldo e RPMI; (G) Inserção, com auxílio de pipeta multicanal, de 100 µl de cada concentração de HMP diluído em BHI caldo ou RPMI + 100 µl da suspensão de *S. mutans* em BHI caldo ou de *C. albicans* em RPMI ; (H) Plaqueamento do conteúdo de cada poço após 48 horas de incubação para determinação da concentração bactericida mínima de HMP.

ANEXO D

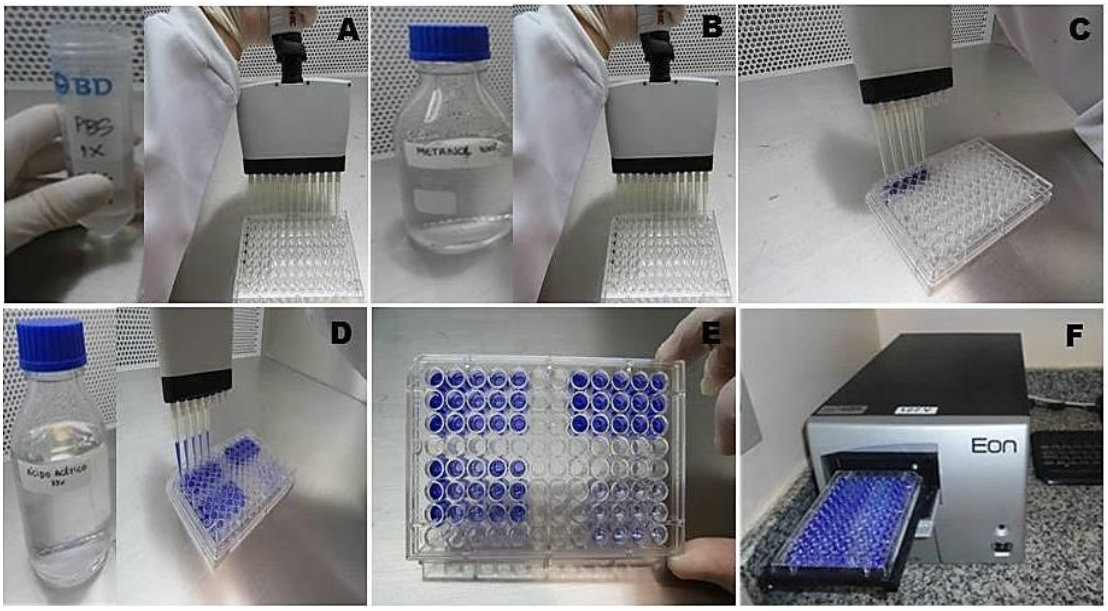
QUANTIFICAÇÃO DAS UNIDADES FORMADORAS DE COLÔNIAS (UFCS) DOS BIOFILMES



(A) Remoção do tratamento e lavagem com NaCl (B) Raspagem do poço com auxilio de ponteiras (200 µL de NaCl 0,85 %) – 5 vezes; (C) Diluições decimais seriadas das suspensões de biofilmes em NaCl; (D), (E) e (F) Plaqueamento das diluições no meio de cultura CHROMagar (*C. albicans*) e BHI ágar (*S. mutans*); (G) e (H) Crescimento das UFCs de *S. mutans* e *C. albicans*, respectivamente.

ANEXO E

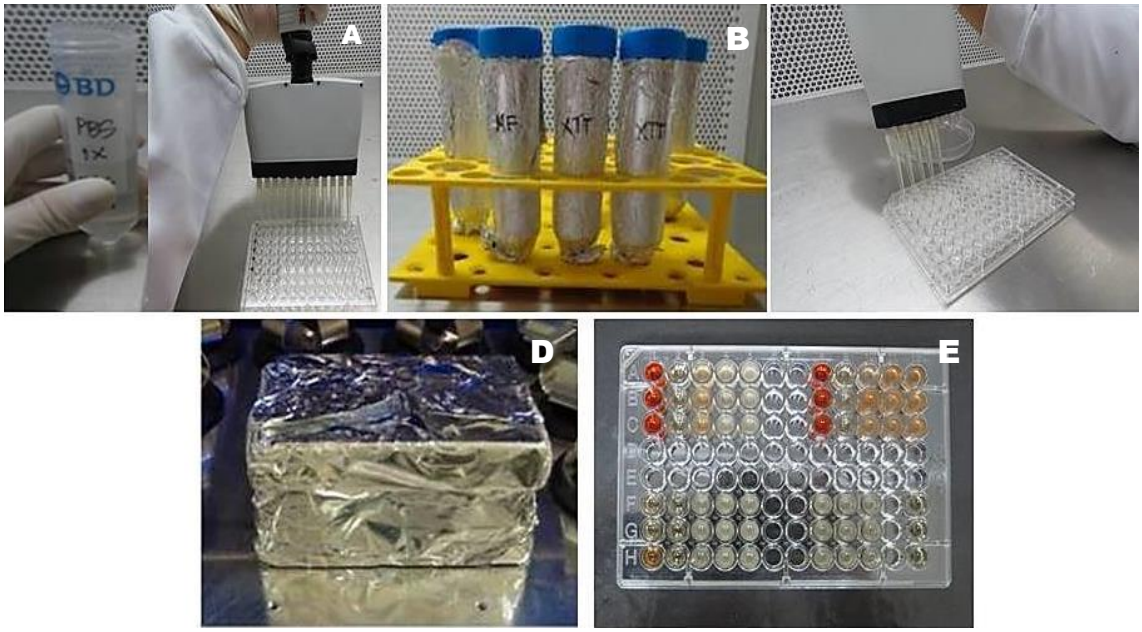
QUANTIFICAÇÃO DA BIOMASSA TOTAL DOS BIOFILMES



(A) Lavagem dos poços com NaCl 0,85% para remoção das células não aderidas; (B) fixação dos biofilmes com metanol 99 %; (C) adição de 200 µL de cristal violeta 1 % para corar os biofilmes; (D) remoção do cristal violeta e adição de 200 µL de ácido acético 33 %; (E) transferência de 200 µl da solução final obtida para poços de placas de microtitulação de 96 poços; (F) leitura de absorbância (570 nm) da solução obtida.

ANEXO F

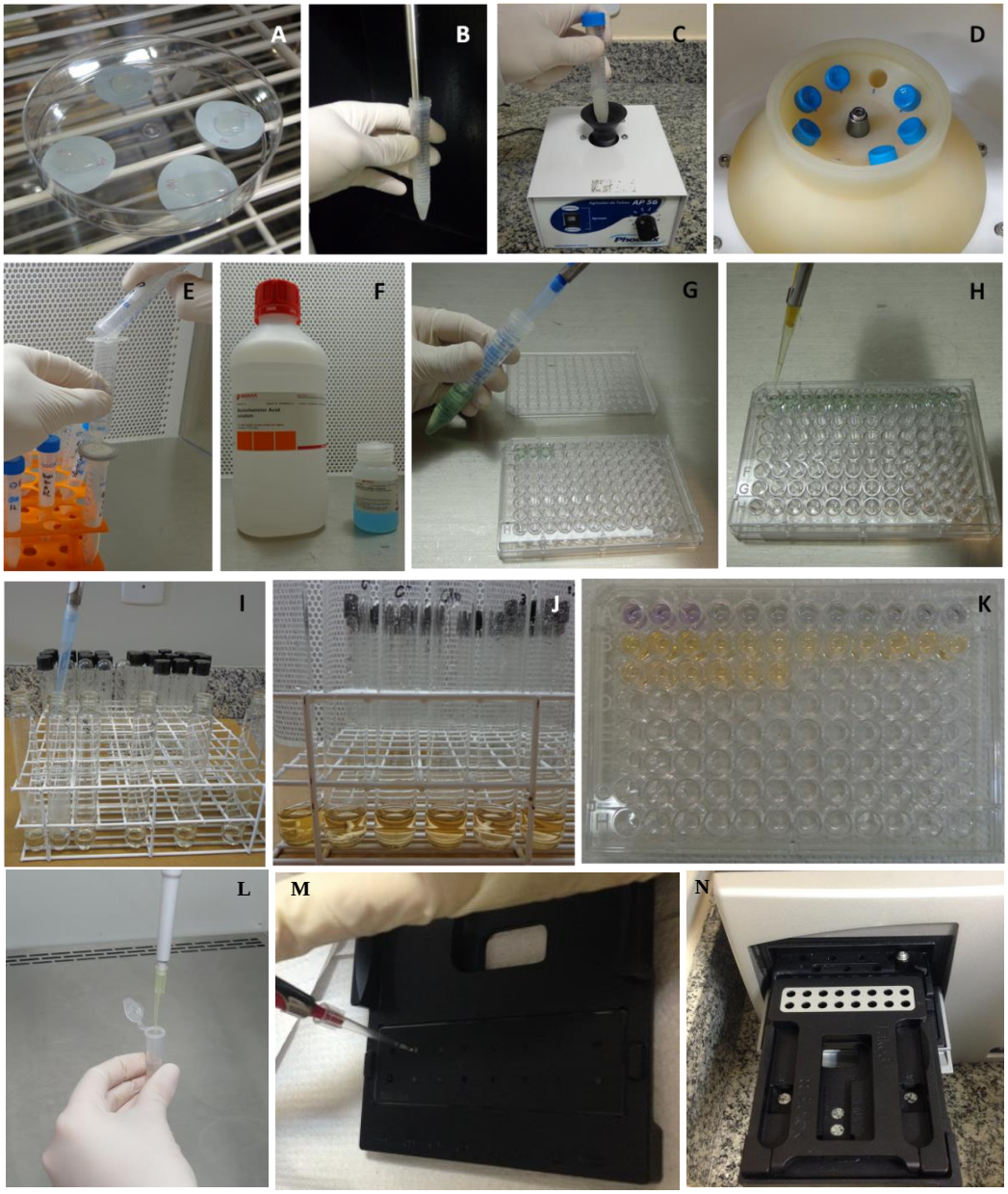
AVALIAÇÃO DA ATIVIDADE METABÓLICA DAS CÉLULAS DOS BIOFILMES



(A) Lavagem dos poços contendo biofilme tratado com 200 µL de NaCl 0,85% (B) soluções de XTT e metassulfato de fenazina (MF) protegidas da luminosidade; (C) incubação das placas com solução de XTT + MF (1 mL/poço) (protegidas da luminosidade) por 3 horas a 37 °C; (D) Diferentes tonalidades (dependendo da maior ou menor atividade metabólica das células) da solução de XTT após o período de incubação; (E) Transferência de 200 µl da solução final para poços de placas de microtitulação de 96 poços para leitura de absorbância (490 nm).

ANEXO G

QUANTIFICAÇÃO DE PROTEÍNA, CARBOIDRATO E ÁCIDOS NUCLEICOS DA MATRIZ EXTRACELULAR DOS BIOFILMES

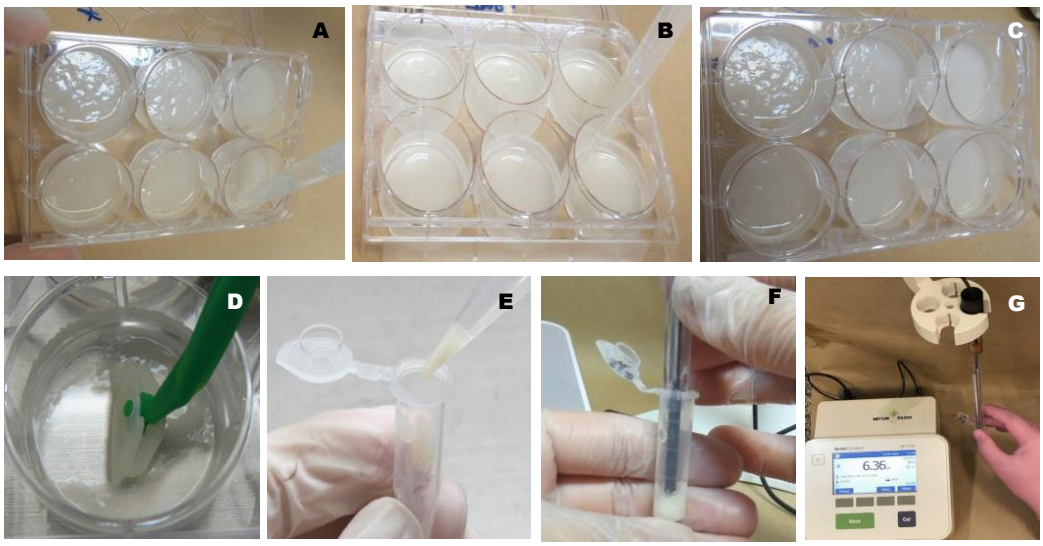


(A) Secagem das amostras de biofilmes após tratamento com HMP e/ou F em membrana de acetato de celulose (previamente pesada), para determinação do peso seco dos biofilmes; (B) Amostra sonicada a 30 w por 30 s e (C) homogeneizada em vórtex; (D) centrifugação das amostras durante 10 min a 3000 *g*; (E) filtragem do sobrenadante obtido anteriormente; (F) Kit BCA utilizado para quantificação do conteúdo proteico da matriz extracelular; (G) Pipetagem de 200 µl da mistura dos reagentes do kit em poços

de placa de 96 poços; (H) Pipetagem de 25 µl do sobrenadante da amostra de biofilme para quantificação de proteínas; (I) Para determinação do conteúdo de carboidrato da matriz extracelular, 500 µl do sobrenadante das amostras foram adicionados à mistura de 500 µl de fenol a 9% e 2,5 mL de ácido sulfúrico em tubos de ensaio de vidro; (J) Diferentes tonalidades de cores nos tubos demonstrando diferentes teores de carboidrato; (K) As quantidades de proteína e carboidrato da matriz dos biofilmes foram determinadas colorimetricamente por leitura em espectrofotômetro a 562 e 490 nm, respectivamente; (L), (M) e (N) Volume de 1,5 µl da fase líquida da matriz extracelular será analisado espectrofotometricamente (260 e 280 nm).

ANEXO H

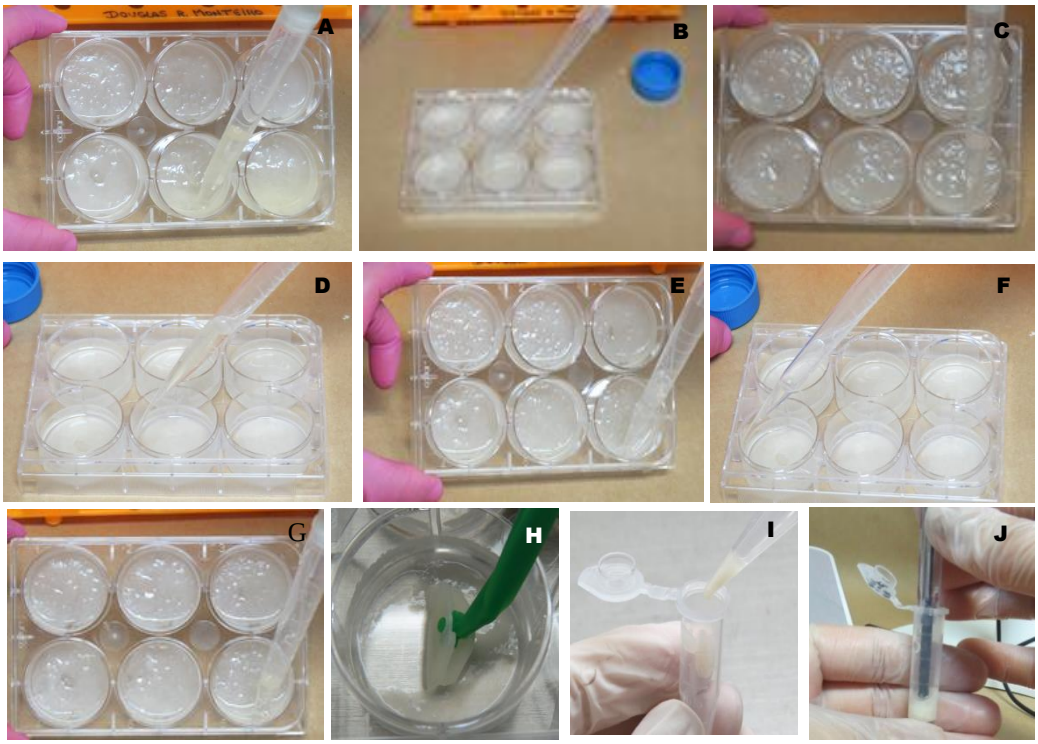
EXPOSIÇÃO À SACAROSE E MEDIÇÃO DE pH



(A) Remoção do meio de cultura (saliva artifical), após 96 h de formação de biofilme; (B) Inserção da solução de sacarose a 10, 20 e 30%; (C) Remoção da sacarose após 1, 3 ou 5 min; (D) Remoção dos biofilmes dos poços utilizando um raspador de células no tempo (t0), e em 1 (t1), 3 (t3), 5 (t5) e 10 (t10) minutos após a remoção da sacarose; (E) Transferência do biofilme para microtubos; (F) e (G) Medição do pH do biofilme antes a exposição e após sacarose.

ANEXO I

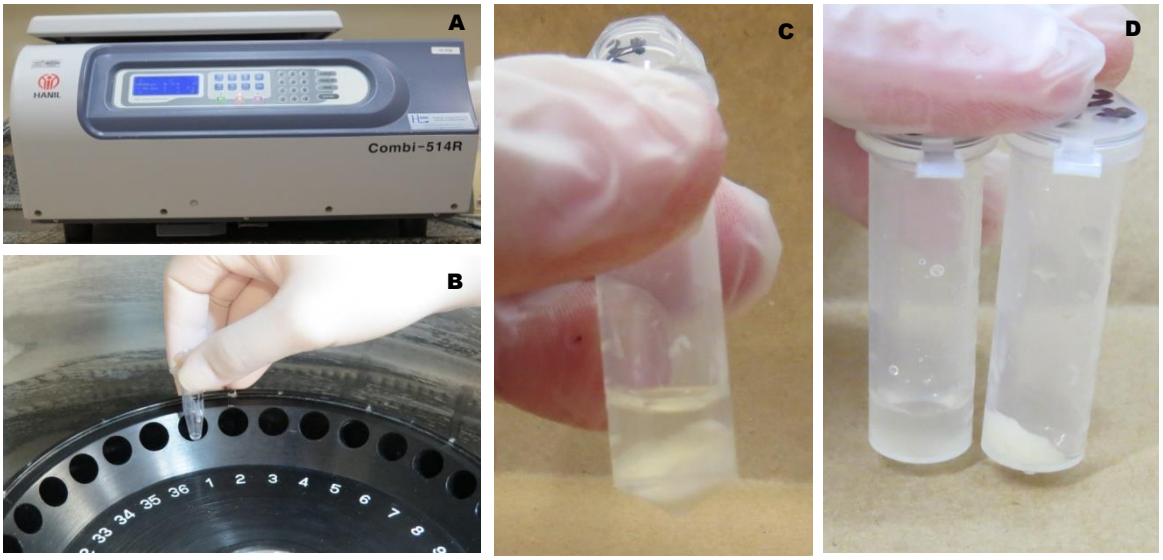
TRATAMENTO E MEDIÇÃO DO pH DOS BIOFILMES



(A) Remoção do meio de cultura (saliva artifical); (B) Tratamento com as soluções; (C) remoção do tratamento; (D) Introdução de saliva artificial (2 mL); (E) remoção da saliva artificial; (F) Solução de sacarose a 20%; (G) Remoção da sacarose após 3 minutos; (H) Raspagem dos biofilmes dos poços utilizando um raspador de células após 1 minuto da remoção da sacarose; (I) Biofilmes transferidos para microtubos; (J) pH do biofilme depois da exposição à sacarose.

ANEXO J

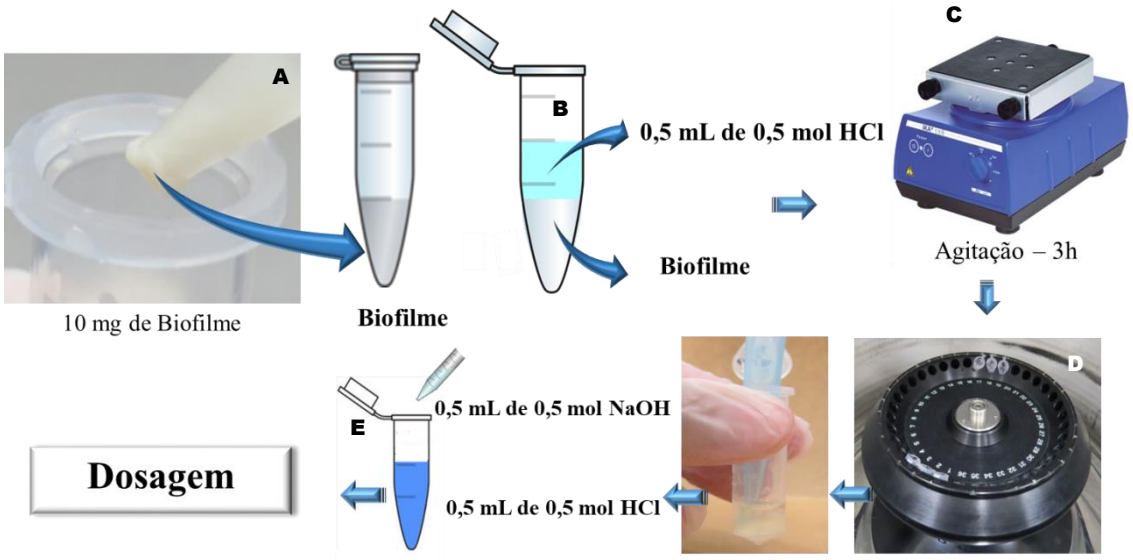
SEPARAÇÃO DO FLUIDO DO BIOFILME



(A) e (B) Centrifugação dos microtubos (1,5267 x g á 4°C por 5 minutos); (C) Aspecto do biofilme após a centrifugação (biomassa e fluido); (D) Separação do fluido com biofilme.

ANEXO K

EXTRAÇÃO DA COMPOSIÇÃO INORGÂNICA DA BIOMASSA



(A) Pesagem de 10 mg de biofilme; (B) Adicionado 0,5 mol/L de HCl aos microtubos contendo os biofilmes na proporção de 0,5 mL/10,0 mg de peso úmido da placa e homogeneizados; (C) Mistura resultante mantida por 3 horas à temperatura ambiente e sob agitação constante (120 rpm); (D) Centrifugação (11.000 × *g*) por 1 minuto; (E) Remoção de quantidade conhecida do líquido e adicionado o mesmo volume de NaOH a 0,5 mol/L.

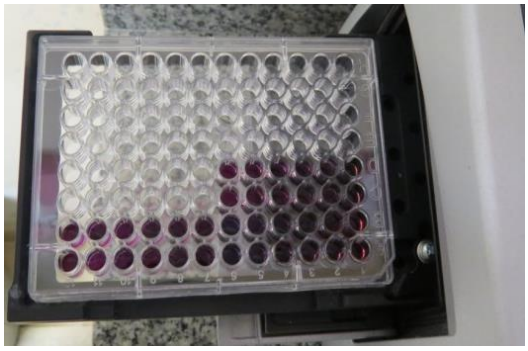
ANEXO L

DOSAGEM DE F NO FLUIDO E NO BIOFILME

(A) Eletrodo específico (Orion 9409 BN) e eletrodo de referência (Orion 900100), ambos acoplados a um potenciômetro (Orion - Thermo Scientific); (B) Proporção tampão (TISAB II) e amostra de 1:1 (40µL de TISABII + 40µL de AMOSTRA) pipetada na interface dos eletrodos para dosagem.

ANEXO M

DOSAGEM DE Ca DO FLUIDO E NO BIOFILME



5 µL da amostra + 50 µL de água deionizada + 50 µL de Arsenazo

Placas foram agitadas por 60 segundos - reação entre a amostra e Arsenazo III

Leitura da absorbância a 650 nm

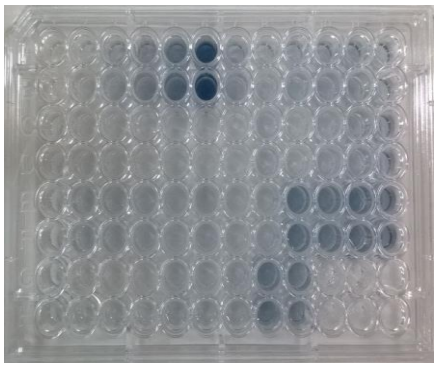
DOSAGEM DE Pi DO FLUIDO E NO BIOFILME

100 µL da amostra + 50 µL de Molibdato de Amônia + 20 µL Reativo Redutor

Placas foram agitadas por 10 minutos - reação entre a amostra e Mobilidato de amônia + 20 min – reação com Reativo Redutor

Leitura da absorbância a 660 nm

DOSAGEM DE P PROVENIENTE DO HMP DO FLUIDO E NO BIOFILME



100 µL da amostra+ 20 µL de Ác. Sulfurico + 50 µL Ác. Periódico + Aquecimento 100°C – 1h

10 µL Sulfito de sódio + 5 µL Molibdato Sódio + 5 µL Hidroquinona - 30 min sem luz

175 µL água deionizada + Leitura da absorbância a 640 nm