



UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE ODONTOLOGIA DE ARAÇATUBA
Departamento de Odontologia Preventiva e Restauradora
Programa de Pós-graduação em Ciências



TESE DE DOUTORADO

Efeitos de nanopartículas de hexametáfosfato de sódio, associadas ou não ao fluoreto, em biofilmes mistos de *Streptococcus mutans* e *Candida albicans* e em biofilmes microcosmos

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CAIO SAMPAIO

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SAMPAIO C. Efeitos de nanopartículas de hexametáfosfato de sódio, associadas ou não ao fluoreto, em biofilmes mistos de *Streptococcus mutans* e *Candida albicans* e em biofilmes microcosmos. 2022. 120 f. Tese (Doutorado em Ciência, área de concentração Saúde Bucal da Criança) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2022.

RESUMO GERAL

O presente estudo avaliou os efeitos de nanopartículas de hexametáfosfato de sódio (HMPnano), associadas ou não ao fluoreto (F), na composição orgânica (Subprojeto 1-S1) e inorgânica (Subprojeto 2-S2) de biofilmes mistos de *Streptococcus mutans* e *Candida albicans* formados *in vitro*; e na viabilidade celular e atividade metabólica de biofilmes microcosmos derivados de saliva (Subprojeto 3-S3). Em S1 e S2, soluções de HMPnano ou HMP microparticulado (HMPmicro) foram preparadas a 0,5% ou 1%, com ou sem F (1100 ppm F, NaF), além de 1100 ppm F (controle positivo) e saliva artificial (controle negativo). *S. mutans* e *C. albicans* foram cultivados em saliva artificial. Os biofilmes foram formados no fundo de poços de placas de microtitulação e tratados 72, 78 e 96 horas após o início da formação, por 1 minuto. Em S1, após o último tratamento, realizou-se análises de quantificação das unidades formadoras de colônias (UFCs), produção de biomassa total, atividade metabólica, além da composição da matriz extracelular dos biofilmes e avaliação estrutural. Observou-se que 1% de HMPnano combinado ao F levou aos menores UFCs de *S. mutans*, bem como às menores concentrações de carboidratos da matriz extracelular dos biofilmes, além de afetar substancialmente a sua estrutura. Em S2, após o último tratamento, avaliou-se o pH e a composição inorgânica dos biofilmes (análise das concentrações de F, cálcio (Ca) e fósforo (P)), antes e após exposição a sacarose. Soluções contendo 1% HMPnano combinado ao F promoveram os maiores valores de pH dos biofilmes, mesmo após exposição à sacarose. Além disso, 1% HMPnano promoveu maiores concentrações de P, enquanto que o HMP (micro/nano, com/sem F) levou a concentrações inexpressivas de Ca no fluido do biofilme. Em S3, os efeitos do HMPmicro ou HMPnano, sozinhos ou associados ao F, foram avaliados em biofilmes microcosmos derivados de saliva. Os biofilmes foram formados sobre discos de vidro por 24 h e, em seguida, *S. mutans* (C180-2) foi incorporado ou não aos biofilmes. A partir deste momento, os mesmos ativos

avaliados em S1/S2 foram adicionados ao meio de cultura, a 20% das concentrações utilizadas nesses subprojetos. Após 96 h de formação, foram determinadas as UFCs totais e de *S. mutans*, e avaliada a produção de ácido láctico pelos biofilmes. Todos os meios de cultura contendo HMP levaram às menores concentrações de ácido láctico e às maiores reduções de UFCs totais e de *S. mutans* dos biofilmes, sem influência do tamanho da partícula de HMP, associação com F ou adição de *S. mutans*. Conclui-se que o HMPnano a 1%, associado a 1100 ppm F, promoveu uma diminuição substancial no metabolismo de biofilmes mistos de *S. mutans* e *C. albicans*, e da viabilidade de *S. mutans*. Esta combinação também levou a valores de pH mais próximos do neutro, além de afetar a composição inorgânica destes biofilmes. Para biofilmes microcosmos, o HMP promoveu a diminuição da viabilidade microbiana e acidogenicidade, sem influência, entretanto, do tamanho da partícula de HMP e da presença de F.

Palavras-chave: Fosfatos; Fluoretos; Biofilmes; Nanotecnologia.

SAMPAIO C. Effects of sodium hexametaphosphate nanoparticles, combined or not with fluoride, on dual-species biofilms of *Streptococcus mutans* and *Candida albicans* and on microcosm biofilms 2022. 120 f. Tese (Doutorado em Ciências, área de concentração Saúde Bucal da Criança) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2022.

GENERAL ABSTRACT

This study evaluated the effects of sodium hexametaphosphate nanoparticles (HMPnano), combined or not with fluoride (F), on the organic (Subproject 1-S1) and inorganic (Subproject 2-S2) compositions of dual-species biofilms of *Streptococcus mutans* and *Candida albicans* formed *in vitro*; and on the cell viability and metabolic activity of saliva-derived microcosms biofilms (Subproject 3-S3). In S1 and S2, solutions containing HMPnano or conventional/micrometric HMP (HMPmicro) were prepared at 0.5 or 1%, combined or not with F (1,100 ppm F, as NaF). Also, a solution containing 1,100 ppm F and pure artificial saliva were tested as positive and negative controls, respectively. *S. mutans* and *C. albicans* strains were cultivated in artificial saliva. The biofilms were formed in well plates, and treated with the test solutions at 72, 78 and 96 from the beginning of the biofilm formation, for 1 minute. In S1, after the last treatment, the number of the colony-forming units (CFUs), production of total biomass, metabolic activity, composition of the extracellular matrix, and the structure of the biofilms were determined. HMP at 1% combined with F led to the lowest *S. mutans* CFUs and lowest concentration of carbohydrates from the extracellular matrix of the biofilms, besides substantially affecting biofilm's structure. In S2, after the last treatment, the pH and the inorganic composition of the biofilms (analysis of F, calcium (Ca), and phosphorus (P) concentrations), prior to and after sucrose exposure, were evaluated. Solutions containing 1% HMPnano combined with F led to the highest biofilm pH, even after exposure to sucrose. In addition, 1% HMPnano promoted the highest P concentrations, while HMP (micro/nano, with/without F) led to inexpressive Ca levels in the biofilm fluid. In S3, the effects of HMPmicro or HMPnano, alone or associated with F, were evaluated in saliva-derived microcosm biofilms, which were formed during 24 h attached to glass coverslips. Thereafter, *S. mutans* (C180-2) was incorporated to the biofilms. From that timepoint onwards, the same actives analyzed in S1/S2 were added to the culture medium at 20% of the concentrations used in those subprojects. After 96 h of formation, total and *S.*

mutans CFU-counting were determined, and the production of lactic acid was assessed. All HMP-containing culture media led to the lowest lactic acid concentrations, and to the highest reductions in total and *S. mutans* CFU counts, with no significant influence of HMP's particle size, association with F or the addition of *S. mutans*. In summary, it was concluded from S1 and S2 that 1% HMPnano combined with 1,100 ppm F substantially reduced the metabolism of *S. mutans* and *C. albicans* dual-species biofilms, besides reducing the viability of *S. mutans*. Also, this combination led to biofilm pH values closer to neutral ones, besides affecting their inorganic composition. From S3, HMP promoted reductions in the microbial viability and acidogenicity, without the influence, however, of HMP's particle size or the presence of F.

Keywords: Phosphates; Fluorides; Biofilms; Nanotechnology.

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INTRODUÇÃO GERAL

A cárie dentária é uma doença dinâmica, multifatorial, mediada por biofilme, modulada pela dieta, não transmissível, determinada por fatores biológicos, comportamentais, psicossociais e ambientais (Pitts et al., 2017; Machiulskiene et al., 2020). Concisamente, uma dieta rica em carboidratos fermentáveis associada à remoção/desorganização ineficiente de biofilme favorece a instalação e progressão da cárie, resultando na perda progressiva de tecidos duros dentais e, quando não interrompida ou controlada, na perda dental (Sheiham & James, 2015).

Biofilmes podem ser definidos como comunidades microbianas amplamente organizadas, aderidas à superfícies vivas ou inertes, e envolvidas por uma matriz extracelular produzida pelas células (Costerton, Stewart, Greenberg, 1999). Esta matriz consiste em um material extracelular, constituído, principalmente, por polissacarídeos, proteínas, ácidos nucleicos e lipídios, os quais conferem ao biofilme estabilidade mecânica, adesão à superfícies, além de atuar como um sistema digestivo externo, mantendo as enzimas extracelulares próximas das células, permitindo-lhes metabolizar substâncias dissolvidas, coloidais, e biopolímeros sólidos (Flemming, 2016). Ademais, a matriz extracelular permite a estabilização de células microbianas no biofilme, estabelecendo interações espaciais estáveis, o que não é possível de ser verificado em condições planctônicas (Flemming et al., 2021). Especificamente quanto ao biofilme dental, além da matriz extracelular, este possui sua porção líquida denominada fluido do biofilme, o qual exerce papel crucial na dinâmica da manutenção da integridade de estruturas minerais dentais, uma vez que a composição do fluido do biofilme determinará a propensão à perda ou ao ganho mineral (Margolis & Moreno, 1992; Fejerskov, 2004). Nesse sentido, o desequilíbrio de componentes inorgânicos entre superfície dental e fluido do biofilme é um fator importante para a determinação do processo cariogênico.

Nas últimas décadas, *Streptococcus mutans* tem sido amplamente estudado e associado com a instalação e progressão da cárie dentária; tal fato se deve, principalmente, à sua habilidade de colonizar a superfície dental, produzir ácido lático a partir da metabolização de carboidratos fermentáveis e, sobretudo, pela sua capacidade de produzir glicosiltransferases (GTFs) (Bowen & Koo, 2011; Lemos et al., 2019). Em suma, especialmente na presença de sacarose, as bactérias se aderem à superfície do dente como resultado da produção de exopolissacarídeos, por meio da atividade das GTFs, fazendo com que o acúmulo de biofilme favoreça a metabolização de sacarose (açúcar ou

polímeros, *e.g.*, amido) por *S. mutans*, gerando, como subproduto, grande quantidade de ácido láctico, o qual é capaz de solubilizar o componente mineral do dente e iniciar o processo cariogênico (Lamont et al., 2006; Marsh & Martin, 2009).

Em acréscimo ao papel inquestionável do *S. mutans* para a cárie dentária, os processos de instalação e progressão da doença também envolvem outros microorganismos (Hajishengallis et al., 2017). Entre eles, destaca-se *Candida albicans*, que é o fungo mais comumente detectado nas superfícies bucais humanas em condições patológicas, atuando em importantes fatores etiopatogênicos, especialmente, na aderência de outros microorganismos, interferindo na sua patogenicidade (Montelongo-Jauregui & Lopez-Ribot, 2018; Cui et al., 2021). Diversos estudos têm relatado a importância de *C. albicans* na instalação e progressão da cárie dentária, os quais têm apontado uma abundante concentração deste microorganismo principalmente em casos de cárie da primeira infância (Falsetta et al., 2014; Xiao et al., 2018; Pereira et al., 2018; Cui et al., 2021). A presença deste fungo em biofilmes contendo *S. mutans*, além de contribuir para o crescimento e desenvolvimento bacteriano, favorece a atividade das GTFs, induzindo a expressão de importantes fatores de virulência desta bactéria como acidogenicidade e adesão (Falsetta et al., 2014; Xiao et al., 2018; Liu et al., 2022).

Evidências robustas associam o efeito de produtos contendo F sobre o controle da cárie, em especial, dos dentifrícios fluoretados (Marinho et al., 2015; 2016; Walsh et al., 2019). O íon flúor exerce seu efeito por meio da manutenção da concentração de F na saliva devido ao uso frequente dos dentifrícios, e pela formação de produtos da reação deste íon com o esmalte ou a dentina, levando à formação do mineral fluoreto de cálcio (CaF_2) que, quando depositado no biofilme dental em lesões de cáries iniciais, é capaz de reduzir a sua progressão (Cruz & Rølla, 1991). Além disso, a presença do F sobre estruturas dentais favorece a formação de fluorhidroxiapatita ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$), estrutura mais resistente a valores de pH ácidos que a hidroxiapatita ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), uma vez que o pH crítico para a dissolução de fluohidroxiapatita e hidroxiapatita são de ~5,5 e ~4,5, respectivamente (Buzalaf et al., 2011). Além do F, íons fósforo (P) e cálcio (Ca) no biofilme dental exercem papel fundamental nos processos de des- e re-mineralização da estrutura dentária. A presença destes íons no ambiente bucal durante o desafio cariogênico favorecem a remineralização e contribui para o controle de desmineralização dental (ten Cate et al., 1996). Além disso, sabe-se que as concentrações de F, Ca e P no biofilme apresentam uma relação inversa com a incidência de cárie (Shaw et al., 1983), possivelmente devido à liberação destes íons para o fluido do biofilme, causando uma

redução na desmineralização e aumento na remineralização pela supersaturação em relação ao esmalte dental (Tanaka & Margolis, 1999).

Apesar do declínio substancial na prevalência e incidência de cárie dentária no mundo todo (Kassebaum et al., 2015), esta doença ainda representa um tópico negligenciado por diversas comunidades, especialmente em países subdesenvolvidos ou em desenvolvimento (Hugo et al., 2019; Kimmie-Dhansay et al., 2022). Nesse sentido, a investigação de estratégias visando à potencialização dos efeitos preventivos de veículos fluoretados tem sido amplamente fomentada, dentre as quais podem se destacar a suplementação destes veículos com sais de fosfatos. A adição de ciclofosfatos a produtos fluoretados tem demonstrado promover efeito sinérgico sobre a des- e re-mineralização do esmalte dental (Gonçalves et al., 2018), sobre o biofilme dental (Cavazana et al., 2019; 2020; Hosida et al., 2021) e sobre a progressão de lesões de cárie em crianças (Freire et al., 2016).

Um dos ciclofosfatos mais amplamente estudados é o hexametáfosfato de sódio (HMP). Recentemente, estudos avaliando a ação do HMP microparticulado sobre biofilmes mistos de *S. mutans* e *C. albicans* demonstraram que a associação destes fosfatos ao F afetou o pH e composição inorgânica do biofilme (concentrações de F, Ca e P), antes e após sua exposição à sacarose (simulando desafio cariogênico), o que fornece dados adicionais para uma melhor compreensão dos mecanismos de ação destes sais, associados ao F, sobre a dinâmica da cárie dentária (Hosida et al., 2022). Observou-se, também, que a associação HMP+F apresentou efeito antimicrobiano/antibiofilme substancial, visto que esta interferiu na composição orgânica, no metabolismo e na formação de biomassa dos biofilmes, além de afetar a expressão dos componentes da matriz extracelular (*i.e.*, proteínas, carboidratos e ácidos nucleicos) e estrutura dos biofilmes (Hosida et al., 2021).

Além disso, tem sido investigada a utilização de nanopartículas de ciclofosfatos, estratégia que vem se mostrando como uma alternativa promissora para a otimização destes produtos (Dalpasquale et al., 2017; Danelon et al., 2019). Estudos *in vitro* (Dalpasquale et al., 2017) e *in situ* (Garcia et al., 2018; Danelon et al., 2019) demonstraram que a adição do HMP nanoparticulado (HMPnano) a um dentifrício convencional (1100 ppm F) promoveu um efeito protetor superior contra a desmineralização do esmalte, além de potencializar a ação remineralizadora deste produto, quando comparado a um dentifrício fluoretado convencional, suplementado ou não com HMP microparticulado (HMPmicro). Ademais, observou-se *in situ* que a adição

de HMPnano a um dentifrício convencional promoveu uma menor produção de polissacarídeos extracelulares (EPS) se comparados aos grupos tratados somente com dentifrícios convencionais, contendo ou não HMPmicro (Garcia et al., 2018). Tais efeitos se devem às propriedades das nanopartículas, como uma maior proporção área/volume, além de maior porcentagem de átomos, o que lhes confere maior reatividade quando comparadas às partículas convencionais/micrométricas (Xu et al., 2010; Cao & Wang, 2011; Jandt & Watts, 2020).

Considerando as evidências supracitadas acerca dos efeitos do HMPnano sobre o esmalte dental, além da escassez de dados sobre a ação desta nanopartícula sobre o biofilme dental, informações adicionais avaliando os efeitos do HMPnano, associado ou não ao F, sobre biofilmes cariogênicos formados sob condições controladas, especialmente envolvendo métodos analíticos complementares aos utilizados nos estudos supracitados (Dalpasquale et al., 2017; Garcia et al., 2018; Danelon et al., 2019) poderia trazer informações importantes acerca do mecanismo de ação deste nanocomposto sobre biofilmes. Neste sentido, este estudo teve o objetivo de verificar os efeitos do HMPnano, associado ou não ao F, sobre: (1) biofilmes mistos de *S. mutans* e *C. albicans* formados *in vitro*, por meio da quantificação de células cultiváveis, biomassa total, avaliação da atividade metabólica, avaliação dos componentes da matriz extracelular e análise estrutural por meio de microscopia eletrônica de varredura (Subprojeto 1); (2) pH e concentrações de F, Ca e P em biofilmes mistos de *S. mutans* e *C. albicans* (biofilme total e fluido do biofilme) formados *in vitro*, antes e após exposição à sacarose (Subprojeto 2); e (3) biofilmes microcosmos, por meio da produção de ácido láctico, quantificação de células cultiváveis e pH dos biofilmes (Subprojeto 3).

Os resultados dos três subprojetos estão apresentados na forma de três capítulos:

Capítulo 1. Effects of nano-sized sodium hexametaphosphate on the viability, metabolism, matrix composition, and structure of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*. Artigo publicado no periódico **Biofouling** (doi: 10.1080/08927014.2022.2064220).

Capítulo 2. Buffering capacity and effects of sodium hexametaphosphate nanoparticles and fluoride on the inorganic components of cariogenic-related biofilms *in vitro*. Artigo publicado no periódico **Antibiotics** (doi: 10.3390/antibiotics11091173.).

Capítulo 3. Effects of sodium hexametaphosphate microparticles or nanoparticles on the growth of saliva-derived microcosm biofilms. Artigo publicado no periódico **Clinical Oral Investigations** (doi: 10.1007/s00784-022-04529-3).

CAPÍTULO 1

Effects of nano-sized sodium hexametaphosphate on the viability, metabolism, matrix composition, and structure of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

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Running head: Nano-sized sodium hexametaphosphate on biofilms.

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Effects of nano-sized sodium hexametaphosphate on the viability, metabolism, matrix composition, and structure of dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

Abstract

This study evaluated the effects of micrometric or nano-sized sodium hexametaphosphate (HMPnano), combined or not with fluoride (NaF, 1100 ppm), on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*. Biofilms were treated with solutions containing the polyphosphates at 0.5 or 1.0%, with/without fluoride (F), in addition to positive and negative controls. Biofilms were analysed by colony-forming units (CFU) counting, metabolic activity, production of biomass, composition of extracellular matrix, and structure. 1% HMPnano+F led to the lowest *S. mutans* CFU, while *C. albicans* CFU counts were not affected by any solution. 1% HMPnano led to the lowest metabolic activity, except for 1% HMPnano+F. All solutions promoted reductions in biofilm biomass compared to controls. Also, 1% HMPnano+F promoted the lowest concentrations of carbohydrates in the biofilm matrix, besides substantially affecting biofilms' structure. In conclusion, HMPnano and F promoted higher antibiofilm effects compared with its micrometric counterpart for most of the parameters assessed.

Keywords: Phosphates; Fluorides; Biofilms; *Streptococcus mutans*; *Candida albicans*; Nanotechnology.

Introduction

Dental caries is a dynamic disease resulting from an imbalance between sugar consumption and biofilm accumulation, determined by biological, behavioural, psychosocial, and environmental factors (Machiulskiene et al. 2020). The progression of dental caries can lead to dental destruction and further tooth loss (Machiulskien et al. 2020). Among the main microorganisms related to caries, the Gram-positive bacteria *Streptococcus mutans* has been highlighted and broadly studied over the years, due to its capacity to form caries-related pathogenic biofilms, resulting from its acidogenic and aciduric traits (Marsh and Martin 2009). Nonetheless, despite the unquestionable role of *S. mutans* on dental caries, the aetiopathogeneses of this disease also comprises the activity of other microorganisms, and another species closely related to dental caries is the fungus *Candida albicans*, especially in early childhood caries (Xiao et al. 2018). Recent studies have demonstrated the relationship between *C. albicans* and *S. mutans* on dental caries dynamics, showing that, besides the onset and progression, the presence of this fungus is intimately associated with caries recurrence (Alkhars et al. 2021, Garcia et al. 2021).

Although caries incidence and prevalence have declined worldwide, untreated caries in permanent teeth persists as the most prevalent health condition all over the world, affecting ~2.4 billion people in the permanent dentition and ~621 million people in the primary dentition (Kassebaum et al. 2015). In this sense, strategies to improve the efficacy of products containing fluoride (F) have been extensively assessed in the last decades, such as the supplementation of such F-products with polyphosphate salts.

Sodium hexametaphosphate (HMP) is an inorganic cyclophosphate salt ((NaPO₃)₆) that presents remarkable synergistic activity with F on caries-related variables. Studies have demonstrated the effects of micrometric/conventional HMP (HMPmicro) on enamel de- and re-mineralisation processes *in vitro* and *in situ* (da Camara et al. 2015, 2016). In addition, the antibiofilm effect of this phosphate, in combination with F, has been observed in dual-species biofilms of *S. mutans* and *C. albicans*, in which HMP and F affected *S. mutans* viability, metabolism, production of total biomass, structure, and composition of the extracellular matrix of the biofilms analysed (Hosida et al. 2021).

In addition to the above-mentioned findings, the reduction of the size of the particles to nanoscale has been shown to promote encouraging effects on caries-related variables. Nano-sized HMP (HMPnano) demonstrated to enhance the effects of a

conventional dentifrice (*i.e.*, dentifrice containing 1,100 ppm F, as NaF) against enamel demineralisation (Dalpasquale et al. 2017) and to increase its effectivity on the promotion of enamel remineralisation (Danelon et al. 2019) in comparison to a conventional dentifrice supplemented or not with HMPmicro. Furthermore, these promising trends were observed on the extracellular polysaccharides (EPS) production, given that a conventional dentifrice containing HMPnano led to a substantial reduction in the expression of the components of the biofilms analysed in comparison to its counterpart with HMPmicro, combined or not with F (Garcia et al. 2019).

Although the effects of HMPnano on tooth enamel have been broadly evaluated over the last years, to date, information on the effects of this nanoparticle combined with F on cariogenic-like biofilms is still scarce. Thus, this study aimed to evaluate the effects of HMPnano, in combination or not with F, on dual-species biofilms of *S. mutans* and *C. albicans*. The null hypothesis of this study was that HMPnano, with or without F, would not affect the biofilms analysed.

Materials & methods

Processing and characterization of HMPnano

The processing and characterization of nano-sized HMP were performed according to Dalpasquale et al. (2017). In brief, 70 g of sodium hexametaphosphate ($((\text{NaPO}_3)_6)$, Sigma Aldrich CAS 7785-84-4, UK) was ball-milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of hexane. After 48 h, the material was filtered and dried at 75 °C (to allow hexane to evaporate). X-ray diffraction (XRD) was performed to analyse the crystalline structure milled for 48 h (HMPnano). The X-ray diffractograms were obtained using a Shimadzu XRD 6000 equipment with $\text{CuK}\alpha$ radiation source ($\lambda = 1.54056 \text{ \AA}$), voltage 30 kV, and current of 30 mA, from samples in powder form. Measurements were made continuously and 2θ range from 5° to 90° with step scan of 0.02° and a scan speed of 0.2°. min^{-1} . The morphology of micrometric HMP (HMPmicro, not milled) and HMP milled for 48 h (HMPnano) were analysed by scanning electron microscopy (SEM) using a Philips XL-30 FEG equipment operating at 10 kV. The samples were prepared by placing three drops of the dilute dispersion in anhydrous ethanol on carbon-coated copper grids (200 mesh, PELCO® Center-Marked Grids) and dried at room temperature over 24 h.

Growth condition

Strains from the American Type Culture Collection (ATCC) were used for the assays: *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. *C. albicans* colonies previously grown 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 (37 °C). For *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% CO₂ at 37 °C. Thereafter, both *S. mutans* and *C. albicans* cells were recovered by centrifugation (at $5,781 \times g$, for 5 min), then washed twice with 10 ml 0.85% NaCl (Sigma-Aldrich, St Louis, MO, USA). Next, the concentration of fungal cells was set using a Neubauer counting chamber at 10^7 cells/ml, while bacterial cells were spectrophotometrically (640 nm) adjusted in a plate reader (EON Spectrophotometer of EON, Biotek, Winooski, VT, USA) to 10^8 cells/ml in artificial saliva (AS) (Lamfon et al. 2003, Cavazana et al. 2019).

The dual-species biofilms were grown at 5% CO₂ (37 °C) in AS supplemented with sucrose according to the following composition for 1 l demi-water: sucrose (4 g), yeast extract (2 g), bacteriological peptone (5 g), mucin type III (1 g), NaCl (0.35 g), CaCl₂ (0.2 g), and KCl (0.2 g), at pH 6.8 (Cavazana et al. 2018). All components for AS preparation were obtained by Sigma-Aldrich (St Louis, MO, USA). The growth medium (*i.e.*, AS) was replenished once daily by removing half of the content of the wells (*i.e.*, 100 µl for biofilms formed in 96-well plates, 1 ml for biofilms formed in 24-well plates, or 2 ml for biofilms formed in 6-well plates) and adding the same volume of a fresh AS.

Treatment of the biofilms

The treatment solutions were prepared at concentrations based on those from previous studies performed aiming to evaluate the effects of HMPmicro and HMPnano on enamel and biofilms (Garcia et al. 2018, Danelon et al. 2019, Hosida et al. 2021), resulting in the following experimental groups: HMP at 0.5% (HMP 0.5), HMP at 1% (HMP 1%), HMP at 0.5%, combined with 1,100 ppm F (HMP 0.5F), HMP at 1%, combined with 1,100 ppm F (HMP 1F), HMPnano at 0.5% (NANO 0.5), HMPnano at 1% (NANO 1), HMPnano at 0.5%, combined with 1,100 ppm F (NANO 0.5F), and HMPnano 1%, combined with 1,100 ppm F (NANO 1F). A solution containing 1,100 ppm F (1100F) was used as the positive control, while AS (CTL) was used as the negative control.

The treatments of the biofilms were performed as proposed by Cavazana et al. (2018), in order to test the actives against mature biofilms. For this, the biofilms were treated at 72 h, 78 h, and 96 h from the beginning of the biofilms formation by gently pipetting the treatment solutions or AS in the wells for 1 min and aspirating them back with a pipette.

Quantification assays

For performing the quantification assays (*i.e.*, colony-forming units counting (CFU-counting), crystal violet assay, and e 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2 tetrazolium hydroxide (XTT) assay), the biofilms were grown in 96-well plates (Costar® #3595, Corning Inc., Corning, NY, USA) at the same conditions described above in the *Growth condition* section, by adding 200 µl of the microbial suspension in the wells. For this, 100 µl of each microbial suspension (2×10^7 cells ml for *C. albicans* and 2×10^8 cells ml for *S. mutans*) were added to the wells (resulting in 200 µl) and the plates were incubated in 5% CO₂ at 37 °C for 72 h.

The number of viable cells was assessed by CFU counting as described elsewhere (Fernandes et al. 2016). In summary, after the last treatment, the biofilms were washed by gently pipetting 200 µl 0.85% NaCl and immediately removing it back. Thereafter, the biofilms were scraped from the wells and resuspended in 0.85% NaCl. After being serially diluted in 0.85% NaCl, the biofilms were plated on BHI agar supplemented with 7 µg ml⁻¹ amphotericin B (Sigma-Aldrich) and on CHROMagar *Candida* (Difco) for further *S. mutans* and *C. albicans* counting, respectively. *C. albicans* plates were incubated aerobically, while *S. mutans* plates were incubated at 5% CO₂ (37 °C), for 24-48 h, at 37 °C. The number of CFU was expressed as Log₁₀ CFU cm⁻².

The total production of biomass by the biofilms was evaluated by the crystal violet (CV) staining assay (Monteiro et al. 2011). Briefly, after the last treatment, the biofilms were fixed for 15 min at room temperature with 99% methanol (Sigma-Aldrich) and stained with 1% CV (Sigma-Aldrich). After 5 min staining, the biofilms were discoloured by exposure to 33% acetic acid (Sigma-Aldrich), and this content was analysed spectrophotometrically in a plate reader (EON Spectrophotometer) at 570 nm. The values were expressed as absorbance cm⁻².

The metabolic activity of the biofilms was assessed by the XTT (Sigma-Aldrich) reduction method (Fernandes et al. 2016). Briefly, XTT was combined with phenazine

methosulphate (Sigma-Aldrich) and gently pipetted into the wells. The plates were then incubated at 37 °C, 120 rpm, for 3 h, and wrapped in aluminium folium for light protection. Thereafter, the content was analysed spectrophotometrically in a plate reader (EON Spectrophotometer) at 490 nm. The values were expressed as absorbance cm⁻².

Evaluation of the composition of the extracellular matrix

For evaluating the composition of the extracellular matrix, dual-species biofilms of *C. albicans* and *S. mutans* were grown in 6-well plates (Costar® #3516, Corning Inc., Corning, NY, USA) at the same conditions as described above in the *Growth condition* section, by adding 4 ml of the microbial suspension in the wells. For this, 2 ml of each microbial suspension (2×10^7 cells ml for *C. albicans* and 2×10^8 cells ml for *S. mutans*) were added to the wells and the plates were incubated in 5% CO₂ at 37 °C for 72 h.

After the last treatment, the biofilms were washed by gently pipetting 4 ml 0.85% NaCl, which was removed immediately afterwards. Thereafter, the biofilms were scraped from the wells, and the liquid phase of the extracellular was extracted by sonication (for 30 s at 30 w), as previously detailed (Cavazana et al. 2019).

For determining the protein content, the bicinchoninic acid method (Kit BCA; Sigma-Aldrich) was adopted using bovine serum as standard (Silva et al. 2009). The concentration of carbohydrates was analysed according to Dubois et al. (1956) using glucose as standard. The concentrations of protein and carbohydrates were expressed as mg g⁻¹ of biofilm dry weight.

Structural analysis of the biofilms

For evaluating the structure of the biofilms, SEM analysis was adopted. For this, dual-species biofilms of *S. mutans* and *C. albicans* were grown in 24-well plates (Costar® #3524, Corning Inc., Corning, NY, USA) at the same conditions as described above in the *Growth condition* section, adding 2 ml of the microbial suspension in the wells. For this, 1 ml of each microbial suspension (2×10^7 cells ml for *C. albicans* and 2×10^8 cells ml for *S. mutans*) were added to the wells and the plates were incubated in 5% CO₂ at 37 °C for 72 h.

After the last treatment, the biofilms were washed by gently pipetting 2 ml 0.85% NaCl, which was removed immediately thereafter. Then, the biofilms were dehydrated using ethanol at increasing concentrations according to the following sequence: 70% for 10 min, 95% for 10 min, and 100% for 20 min, followed by a 20-minute air dry (Silva et

al. 2013). The bottom of the wells was then cut using a scalpel blade (number 11, Solidor, Lamedid Commercial and Services Ltd., Barueri, Brazil) after flame-sterilisation. Thereafter, the biofilms were coated with gold and analysed in an electron microscope (S-360 microscope, Leo, Cambridge, MA, USA). The structure of the biofilms was evaluated in terms of the presence of coccus, hyphae, and yeasts, as well as the structure and density of the biofilms' matrix, at different magnifications. The area analysed was selected by verifying in high magnification the most representative area of the specimen.

Statistical analysis

Data on *C. albicans* CFU-counting, concentration of proteins and carbohydrates of the extracellular matrix passed normality (Shapiro-Wilk) and homogeneity (Barlett) tests and were submitted to 1-way ANOVA, followed by Student-Newman-Keuls' test. Data on *S. mutans* CFU-counting did not pass normality (Shapiro-Wilk) and homogeneity (Barlett) tests, and were therefore submitted to Kruskal Wallis' test, followed by Student-Newman-Keuls' test. Data on biomass production and metabolic activity passed normality (Shapiro-Wilk) and homogeneity (Barlett) tests and were submitted to 1-way ANOVA, followed by Tukey's test. All experiments were performed in triplicate, on three different occasions ($n = 9$). Statistical analyses were performed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA), adopting $p < 0.05$.

Results

Processing and characterization of HMPnano

XRD pattern for HMP shows an amorphous phase, with no changes in the crystalline standard of the particles prior (HMPmicro) or after (HMPnano) nano-synthesis (Figure 1). SEM images show large aggregates particles for HMPmicro (average $2.05 \pm 0.69 \mu\text{m}$) and low-size particles for HMPnano ($0.38 \pm 0.12 \mu\text{m}$) (Figure 2). In addition, both HMPmicro and HMPnano presented an overall trend of spherical particles (Figure 2).

Quantification assays

CFU counts of *C. albicans* were not affected by any solution assessed (Figure 3a). Conversely, HMPnano at 1% + F led to the highest CFU reduction of *S. mutans*, followed by HMP micro at 1% + F and positive control (similar to each other), and the remaining groups (Figure 3b). Furthermore, HMPnano at 1% led to significantly lower metabolic

activity compared to all other groups (except for HMPnano at 1%, combined with F) (Figure 4a). Also, all HMP-containing solutions (HMPmicro or HMPnano), combined or not with F, led to significant reductions in the production of biofilm biomass in comparison to the positive and the negative controls (Figure 4b).

Evaluation of the composition of the extracellular matrix

Treatments with HMPmicro at 1% and HMPnano at 0.5% or 1% led to significant reductions in the concentration of proteins in comparison to the negative control. In addition, HMPnano at 0.5% was the only treatment able to significantly reduce protein concentration in comparison to the positive control (Figure 5a). Regarding the expression of carbohydrates, the highest concentrations were observed for the CTL group, which were significantly different from all other groups. Furthermore, biofilms treated with HMPnano at 1% combined with F, presented the lowest concentrations of carbohydrates in comparison to all other groups (Figure 5b).

Structural analysis of the biofilms

Dual-species biofilms treated with HMP at 0.5% at both particle sizes and those from the CTL group presented the densest structure among all groups, which were composed of yeasts and hyphae heavily aggregated with cocci. Conversely, for biofilms exposed to HMPmicro or HMPnano combined with F, or exposed to 1100 ppm F only, a slight tendency of a reduction in density can be observed. The least density was observed for biofilms treated with HMP at 1% at both particle sizes. Such effects were further enhanced when HMP at 1% was associated with F, as biofilms were the least dense among all groups, with reduced amounts of cocci and yeasts (Figure 6).

Discussion

Recent data demonstrated that HMP combined with F promoted remarkable synergistic effects on the organic composition of dual-species biofilms of *S. mutans* and *C. albicans*, mainly affecting the viability of *S. mutans*, and the expression of some components of the extracellular matrix (Hosida et al. 2021). These findings encouraged the development of this study, given that the reduction of the size of the particles to nanoscale could enhance the effects of this association, similar to that observed for variables related to tooth de- and re-mineralisation (Dalpasquale et al. 2017, Garcia et al. 2019, Danelon et al. 2019). The present study demonstrated that HMPnano combined

with F affected several parameters related to the structure, metabolism, and composition of the dual-species biofilms analysed, leading thus to the rejection of the study's null hypothesis.

Treatments with solutions containing HMP at both concentrations and particle sizes, regardless of the presence of F, led to significant reductions in biofilm biomass production, compared to positive and negative controls, with no evident trend among the HMP-containing solutions. Similar findings were observed for the metabolic activity of the biofilms, except that solutions containing HMPnano at 1%, combined or not with F, promoted the lowest values among the other groups. Such reductions can be justified by the great affinity that HMP presents to metal ions, such as Mg^{2+} , Ca^{2+} , K^+ , Fe^{3+} , which favours the formation of ionic complexes with these ions present in the bacterial cell walls, consequently interfering with its permeability (Changgen and Yongxin 1983, Choi et al. 1993). The superior effects observed for the nanoparticles, in line with those observed for enamel de- and re-mineralisation variables (Dalpasquale et al. 2017; Danelon et al. 2019, Garcia et al. 2019), can be possible justified by their physical-chemical characteristics. Briefly, due to their lower size, nanoparticles have a higher surface area/volume ratio, thus leading to an increased reactivity in comparison to conventional/micrometric particles (Xu et al. 2010). This assumption can be further confirmed by observing the results presented on the nanoparticles processing and characterisation, which it was demonstrated a substantial decrease in the particles size of HMP after 48 h ball-milling, and no change in the crystalline standard of the particles.

Despite the promising results described above, none of the test solutions was able to affect the CFU of *C. albicans*, similarly to data previously reported (Hosida et al. 2021). Such a trend may be related to the high resistance of this fungus to conventional therapies, which has been widely reported in the literature (Ding et al. 2021). *C. albicans* consists of a polymorphic microorganism (*i.e.*, able to present itself as yeast, pseudo-hyphae, and hyphae) what is, possibly, the most important virulence factor of this fungi, by affecting immune recognition and pathogenesis, providing to *C. albicans* resistance to conventional drugs (Mukaremera et al. 2017, Khan et al. 2021).

Conversely, the exposure of the biofilms to the solution containing HMPnano at 1% and F led to the lowest CFU number of *S. mutans* in comparison to all the other groups. A direct comparison of this group with HMPmicro at 1% and F clearly shows that the reduction of the particle size further enhanced the effects of the phosphate on the biofilms, achieving superior inhibitory properties in comparison to conventional

HMPmicro and the positive control (only F). Similarly to the observed for biofilms' metabolism and biomass production, the results above can be explained by the antimicrobial capacity of this phosphate due to its chelating ability (Changgen and Yongxin 1983; Choi et al. 1993), while the superiority of the nanoparticles on the production of biofilm biomass can be justified by their higher reactivity (Xu et al. 2010). This trend can be further reinforced by observing the structure of the biofilms, in which 1% HMPnano combined with F resulted in a less dense biofilm, and a reduction in the number of cocci attached to the biofilm.

Concerning treatment with only F (positive control), in line with previous data (Hosida et al. 2021), it has been shown to lead to a discrete significant reduction in the *S. mutans* CFU in comparison to the negative control group. The antimicrobial effect of F *in vitro* can be mainly summed up by its action on biofilm's acidogenicity, acidurance, and adherence to the tooth surface (Liao et al. 2017). Briefly, F penetration into bacterial cytoplasm depends on the hydrogen fluoride (HF) influx, which is then dissociated into protons (H^+) and F ions (F^-) in the cytoplasm; pH drops in the extracellular environment facilitates the association of H^+ and F^- to HF. These ions are known to interfere with the glycolytic via mainly by inhibiting the enzymatic activity of enolase (Hamilton 1990, Marquis et al. 2003, Liao et al. 2017). Thus, the ability of *S. mutans* to stand to repetitive pH challenging is impaired by the presence of F (*i.e.*, reduction of acidurance), especially due to the acidification of the cytoplasm via HF influx, and by the inhibition of proton-extruding F-ATPase (Bender et al. 1986, Marquis 1990). Despite tooth adherence was not addressed in the present work, it is known that F interferes with this variable especially by inhibiting the activity of glucosyltransferases (GTFs) (Pandit et al. 2011), so it is possible that the effects of F on the biofilms could have been more evident by including tooth surfaces for biofilm formation. In this sense, further protocols addressing the biofilm formation adhered to tooth or tooth-like surfaces could bring useful information on the activity of F combined with HMP on the bacterial viability.

Surprisingly, unlike previous data (Hosida et al. 2021), the synergism between F and HMPmicro on *S. mutans* viability was not observed in this study, given that 1% HMPmicro and F led to *S. mutans* viability similar to the positive control group (*i.e.*, 1,100 ppm F). Although the reason for such a trend is not evident, molar ratio considerations can help to justify it: while Hosida et al. (2021) tested HMPmicro combined with F at 500 ppm, in the present study the effects of the phosphates were evaluated in combination with F at 1,100 ppm, as an attempt to further increase the effects

of the phosphate-containing solutions compared with the above-mentioned study. Nonetheless, based on the present data, it seems plausible that the increase in F concentration should be accompanied by an increase in HMP concentrations to keep an optimum molar ratio. In this way, assuming a linear relationship between F and HMP, the combination of 1% HMP and 500 ppm F would be equivalent to ~2% HMP and 1,100 ppm F. The same rationale can be used to justify the lack of effects for the groups treated with F combined with HMPmicro or HMPnano at 0.5%.

In addition to the aforementioned trends, HMPnano at 1% associated with F led to remarkable significant effects on carbohydrate concentrations in the biofilm matrix. This consists of organic polymers surrounding the biofilms, whose main functions include biofilm adhesion, resistance, and biofilm communication and exchange of genetic information (Flemming 2016). Thus, the effects described above may help to explain the favourable results of HMPnano at 1% + F on other parameters analysed, especially *S. mutans* CFUs. This also suggests that the less dense structure of the biofilms exposed to HMPnano at 1% combined with F (SEM images) might have resulted from the lower concentration of carbohydrates. Although the dual-species biofilms used in the present study have a simpler nature in comparison to polymicrobial models, the effects of this nano-polyphosphate and F on these variables encourage further assessment of HMPnano on more complex biofilms at more dynamic conditions, especially considering the important role that the extracellular matrix on caries dynamics (Bowen et al. 2018).

Based on the above, it can be concluded that HMPnano significantly reduced *S. mutans* CFUs, besides interfering with metabolism, production of total biomass, structure, and the composition of the extracellular matrix of these biofilms. The results presented in this study elucidate the effects that this nano-phosphate and F can exert on cariogenic-like biofilms.

Acknowledgments

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Declaration of Interest Statement

No potential conflict of interest was reported by the authors.

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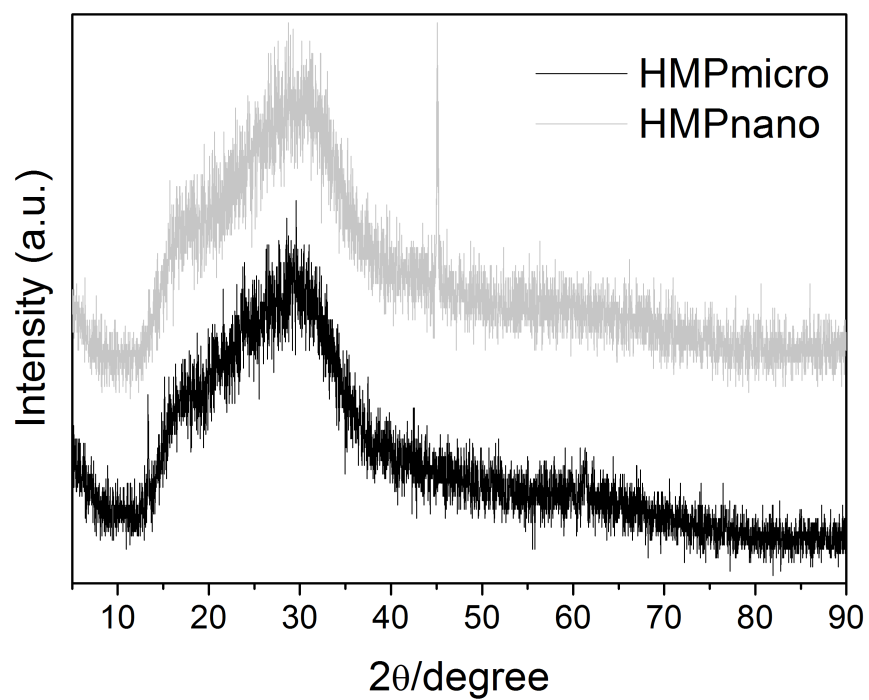
Figures

Figure 1. X-ray pattern of sodium hexametaphosphate prior and 48 h after ball-milling (commercial HMP and nano-sized HMP, respectively).

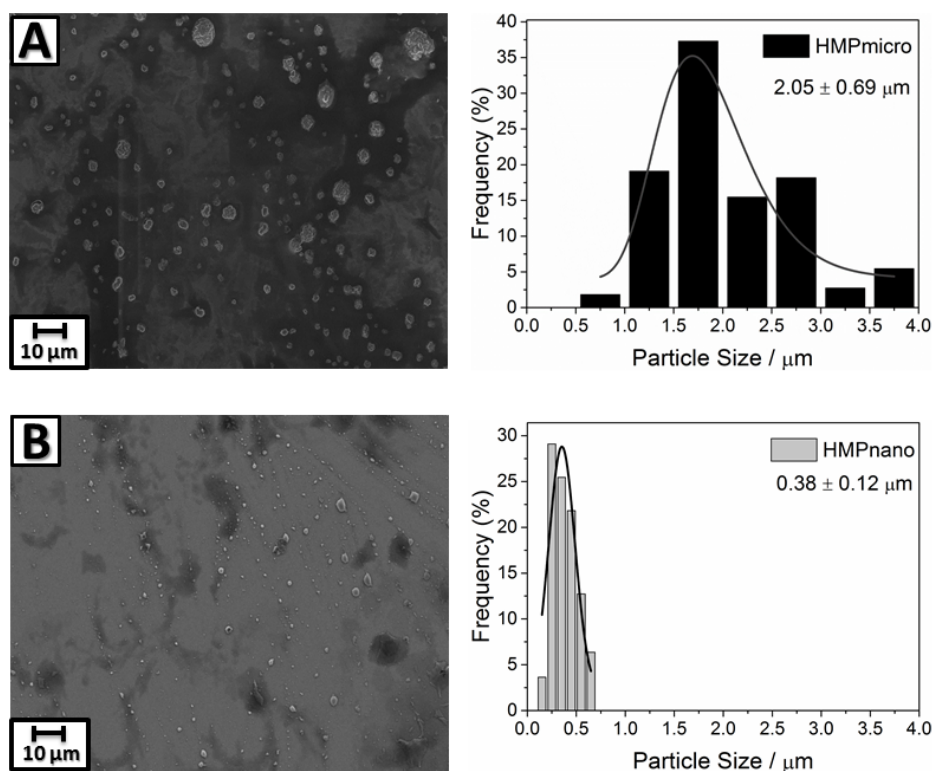


Figure 2. Scanning electron microscopy analysis of (a) commercial sodium hexametaphosphate (HMPmicro) and (b) sodium hexametaphosphate after 48 h ball-milling (HMPnano).

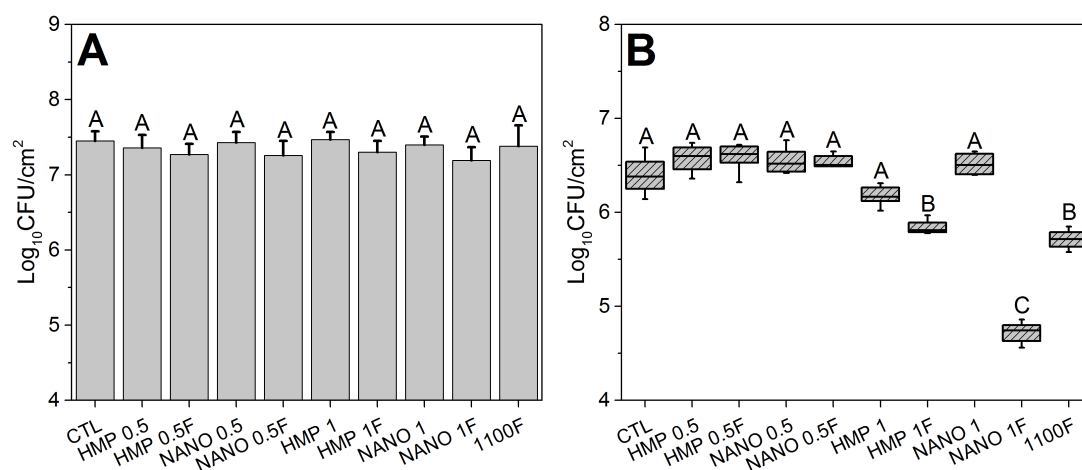


Figure 3. Logarithm of colony-forming units cm^{-2} for *Candida albicans* (a) and *Streptococcus mutans* (b) in dual-species biofilms. Bars denote the standard deviations of the means. Different upper case letters denote statistical differences among the groups. CTL: negative control (artificial saliva); 1100F: 1,100 ppm F; HMP: sodium

hexametaphosphate; NANO: nano-sized sodium hexametaphosphate. Student-Newman-Keuls' test ($p < 0.05$, $n = 9$).

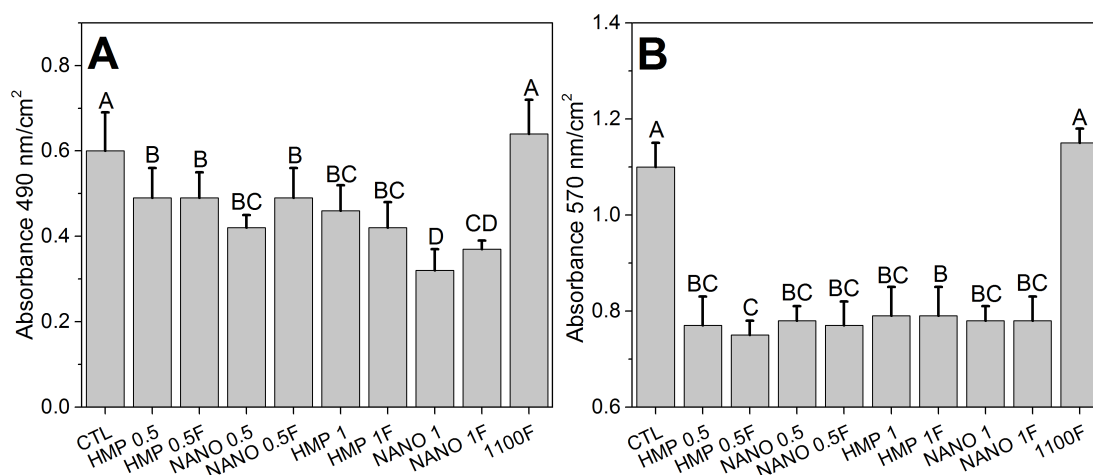


Figure 4. Absorbance/cm⁻² values obtained for the (a) metabolic activity and (b) total biomass quantification assays. Bars denote the standard deviations of the means. Different upper case letters denote statistical differences among the groups. CTL: negative control (artificial saliva); 1100F: 1,100 ppm F; HMP: sodium hexametaphosphate; NANO: nano-sized sodium hexametaphosphate. Tukey's test ($p < 0.05$, $n = 9$).

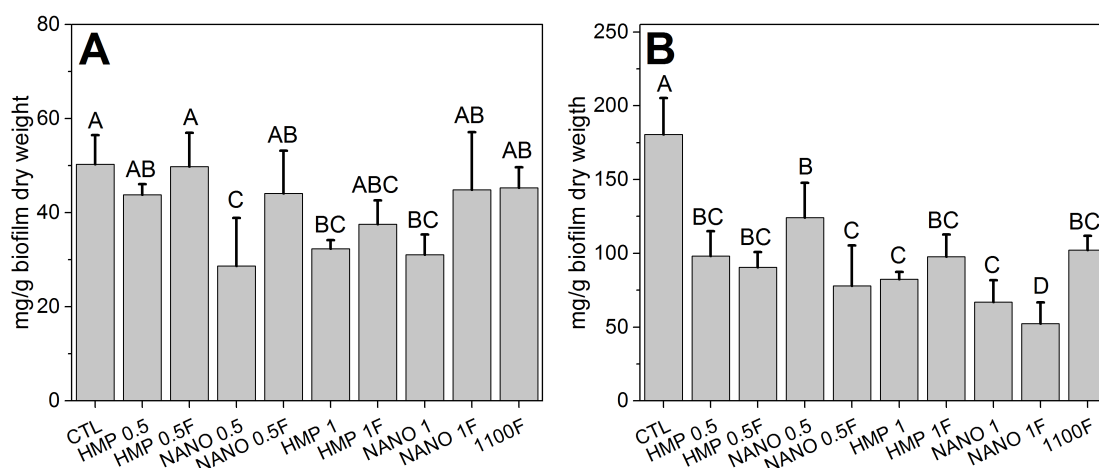


Figure 5. Mean (SD) values of protein (a) and carbohydrate (b) of the extracellular matrix of dual-species biofilms of *Streptococcus mutans* and *Candida albicans* obtained after treatment with different concentrations of HMPmicro or HMPnano, combined or not with F. Different upper case letters denote statistical differences among the groups. CTL: negative control (artificial saliva); 1100F: 1,100 ppm F; HMP: sodium

hexametaphosphate; NANO: nano-sized sodium hexametaphosphate. Student Newman Keuls' test ($p < 0.05$, $n = 9$).

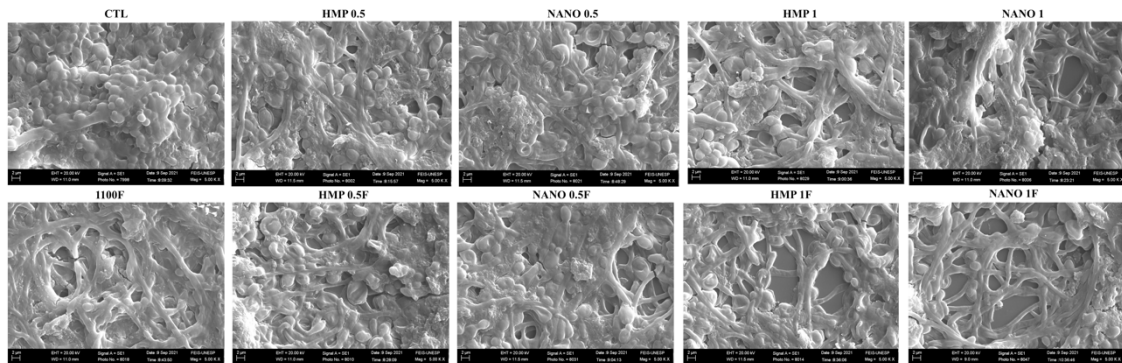


Figure 6. Scanning electron microscopy images of dual-species biofilms of *Candida albicans* and *Streptococcus mutans* after treatment with different experimental solutions. Magnification: $\times 5.0$ k. Bars: 2 μm . CTL: negative control (artificial saliva); 1100F: 1,100 ppm F; HMP: sodium hexametaphosphate; NANO: nano-sized sodium hexametaphosphate.

CAPÍTULO 2

Buffering capacity and effects of sodium hexametaphosphate nanoparticles and fluoride on the inorganic components of cariogenic-related biofilms *in vitro*

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Short title: Sodium hexametaphosphate nanoparticles on biofilms

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Keywords: Biofilms; Fluorides; Nanotechnology; Phosphates.

Abstract

Despite the remarkable effects of sodium hexametaphosphate nanoparticles (HMPnano) on dental enamel de-/re-mineralization processes, information on the effects of these nanoparticles on biofilms is scarce. This study assessed the effects of HMPnano, with or without fluoride (F), on the inorganic components and pH of *Streptococcus mutans* and *Candida albicans* dual-species biofilms. Solutions containing conventional/micro-sized HMP (HMPmicro) or HMPnano were prepared at 0.5% and 1%, with or without 1100 ppm F. A 1100 ppm F solution and pure artificial saliva were tested as positive and negative controls, respectively. The biofilms were treated three times and had their pH analyzed, and the concentrations of F, calcium, phosphorus, and HMP in the biofilm biomass and fluid were determined. In another set of experiments, after the last treatment, the biofilms were exposed to a 20% sucrose solution, and the biofilm pH and inorganic components were evaluated. The 1% HMPnano solution with F led to the highest biofilm pH, even after exposure to sucrose. The 1% HMPnano solution without F led to significantly higher phosphorus concentrations in comparison to all other groups. It can be concluded that 1% HMPnano and F influenced the biofilm pH, besides affecting most of the inorganic components of the dual-species biofilms.

Keywords: Biofilms; Fluorides; Nanotechnology; Phosphates.

1. Introduction

Dental caries is a multifactorial, dynamic disease, mediated by the dental biofilm and modulated by the diet [1], resulting from the acid production by the biofilm from carbohydrate metabolism [2]. Considering the dynamics involved in de- and re-mineralization processes, the pH of the biofilm fluid, as well as the concentrations of fluoride (F), phosphorus (P), and calcium (Ca) in the biofilm (solid and fluid phases) are known to exert determinant role on the saturation of hydroxyapatite [3, 4, 5]. This has important clinical implications, as an inverse relationship between caries incidence and the inorganic composition of saliva and dental biofilm has been demonstrated [6].

The gram-positive bacterium *Streptococcus mutans* plays a crucial role in dental caries onset and progression [7], especially due to its capacity to produce organic acids and to stand to stressful conditions such as low pH. It also has the ability to synthesize large amounts of extracellular polymers from sucrose metabolism, thereby promoting supportive conditions for the adhesion and aggregation of other acidogenic and aciduric microorganisms to the biofilm [8]. In addition to *S. mutans*, recent data have stated the role that the species *Candida albicans* exert in caries dynamics, particularly due to the critical symbiotic activity between *S. mutans* and *C. albicans* [9, 10], in which the production of polysaccharides by *C. albicans* seems to impact the development of *S. mutans* biofilms [9], what increases caries severity.

Despite the decline in caries incidence and prevalence worldwide [11], dental caries substantially affects several individuals and populations all over the world [2]. This has prompted to the investigation of new strategies, including the supplementation of fluoridated products with different compounds, such as sodium hexametaphosphate (HMP), an inorganic cyclophosphate salt, which is known to increase the permeability of the microbial wall and to disperse the biofilm [12, 13]. Recent data evaluating the activity of conventional/micro-sized HMP (HMPmicro) on dual-species biofilms of *S. mutans* and *C. albicans* demonstrated that a solution containing HMPmicro in combination with F substantially affected the inorganic components of the biofilms and promoted an enhanced buffering effect in comparison to a 1,100 ppm F solution [14]. In addition, it was demonstrated that the incorporation of HMP nanoparticles (HMPnano) to fluoridated toothpastes led to superior protective effects against enamel demineralization, and reduced the production of extracellular polysaccharides by biofilms in situ in comparison to fluoridated toothpastes containing HMPmicro [15]. Also, this nanoparticle has been shown to promote substantial reductions in the cell viability, metabolism, and production

of biomass, besides affecting the structure and the composition of the extracellular matrix of a *S. mutans* and *C. albicans* dual-species biofilms [16].

Considering the promising effects of HMPmicro on the inorganic components of biofilms [14], in addition to the substantial protective activity of HMPnano on enamel [15] and on the viability and virulence of cariogenic-related biofilms [16], it would be interesting to evaluate the effects of HMPnano on the pH, and on the F, calcium (Ca), phosphorus (P) and HMP concentrations of biofilms. Thus, the aim of this study was to evaluate the effects of HMPnano, combined or not with F, on the pH and inorganic components of dual-species biofilms of *S. mutans* and *C. albicans*, prior to and after exposure to sucrose. The null hypothesis of this study was that the effect of HMP would not be influenced by its particle size, presence of F, or exposure to sucrose.

2. Results

2.1. Determination of the biofilm pH

All test groups presented significantly higher pH in comparison to the negative control, both prior to and after exposure to sucrose. Also, biofilms treated with HMP or HMPnano combined with F presented significantly higher pH in comparison to their counterparts without F, regardless of the exposure to sucrose. NANO 1/F group showed the highest pH in comparison to all the other groups, followed by the HMP 1/F group (Figure 1). Exposure to sucrose promoted a significant decrease in biofilm pH for all study groups, following a similar trend to that observed for biofilms not exposed to sucrose.

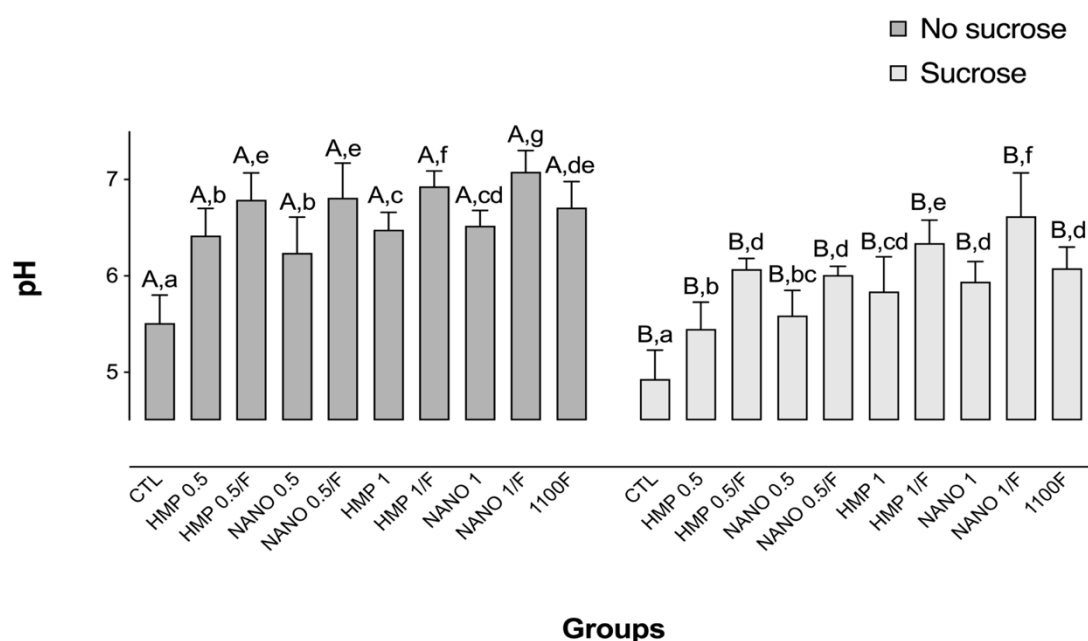


Figure 1. Mean pH values, prior to and after exposure to sucrose (cariogenic challenge). Distinct capital letters indicate statistical difference within each group regarding exposure to a 20% sucrose solution (in two levels – yes or no). Distinct lowercase letters indicate statistical difference among the experimental groups (all test solutions), within each condition of sucrose exposure). Data were submitted to 2-way ANOVA, followed by Fisher's LSD *post hoc* test for multiple comparisons ($p < 0.05$; $n = 9$). Bars denote standard deviations of the means. HMP: sodium hexametaphosphate; CTL: negative control; NANO: nano-sized HMP.

2.2. Analysis of the inorganic composition of the biofilm fluid

For the biofilms not exposed to sucrose, all groups treated with F-containing solutions had significantly higher F levels in comparison to the other groups (Figure 2A). After exposure to sucrose, biofilms treated with HMP 1/F or NANO 1/F presented the highest F concentrations, surpassing those attained by the use of 1100F (Figure 2A). Regarding Ca concentrations, exposure to phosphate-containing solutions led to significantly lower Ca levels in comparison to the negative and positive controls, which was not shown to be dependent on the particle size or exposure to sucrose (Figure 2B). In addition, the negative control group presented the highest Ca concentrations, followed by the positive control (Figure 2B).

Prior to the exposure to sucrose, biofilms treated with phosphate-containing solutions, regardless of particle size, led to significantly higher P concentrations in the

biofilm fluid, in a dose-dependent manner. Furthermore, the association of HMP with F significantly reduced P levels in the biofilm fluid for all pairwise comparisons (Figure 2C). The highest P value was observed for NANO 1, which was significantly different from all the other groups except NANO 1/F (Figure 2C). A similar trend was observed for HMP in the biofilm fluid, both prior to and after exposure to sucrose (Figure 2D).

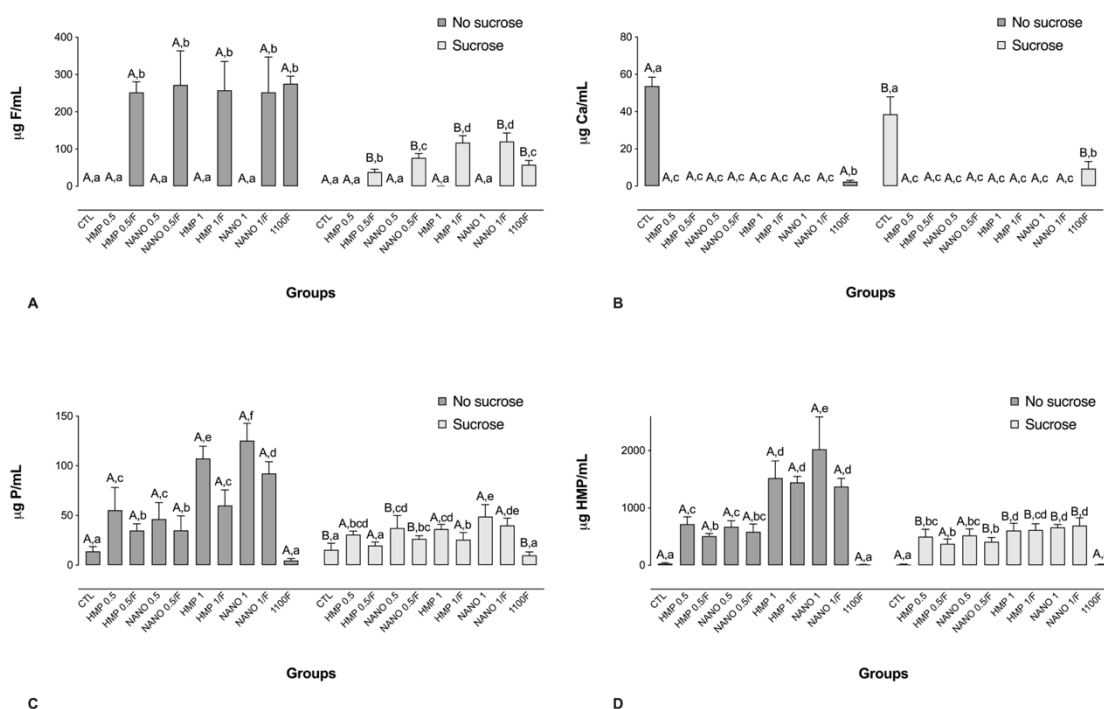


Figure 2. Mean values of F (A), Ca (B), P (C), and HMP (D) in the biofilm fluid, prior to and after exposure to sucrose (cariogenic challenge). Distinct capital letters indicate statistical difference within each group regarding exposure to a 20% sucrose solution (in two levels – yes or no). Distinct lowercase letters indicate statistical difference among the experimental groups (all test solutions), within each condition of sucrose exposure). Data were submitted to 2-way ANOVA, followed by Fisher LSD's *post hoc* test for multiple comparisons ($p < 0.05$; $n = 9$). Bars denote standard deviations of the means. Ca: calcium; F: fluoride; P: phosphorus; HMP: sodium hexametaphosphate; CTL: negative control; NANO: nano-sized HMP.

2.3. Analysis of the inorganic components from the biofilm biomass

Treatment with 1100F led to the highest F levels, followed by treatments with HMPmicro or HMPnano at 1% combined with F, and HMPmicro or HMPnano at 0.5% combined with F. The exposure to sucrose promoted a significant decrease in F levels for

all the F-containing groups (Figure 3A). Regarding Ca concentrations, a similar trend to that seen for the biofilm fluid was noted, in which the highest Ca levels were observed for the CTL, followed by 1100F. Also, prior to the exposure to sucrose, similar Ca levels were observed for all the HMP-containing groups, regardless of the presence of F or the particle size (Figure 3B).

A trend for a dose-response relationship was observed with regard to HMP concentrations of the test solutions and P levels in the biofilm biomass both prior to and after exposure to sucrose. In addition, without sucrose exposure, the addition of F to the HMP-containing solutions significantly reduced P levels in comparison to their counterparts without F (Figure 3C). A similar pattern was observed for HMP concentrations, with a lesser impact of F though (Figure 3D).

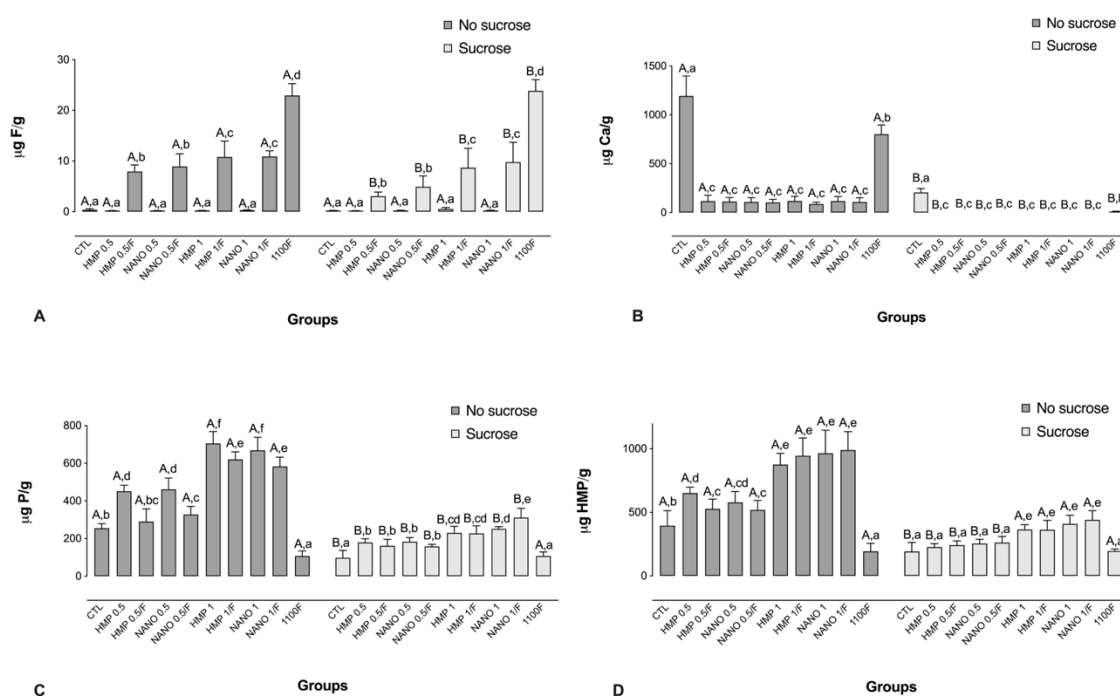


Figure 3. Mean values of F (A), Ca (B), P (C), and HMP (D) in the biofilm biomass, prior to and after exposure to sucrose (cariogenic challenge). Distinct capital letters indicate statistical difference within each group regarding exposure to a 20% sucrose solution (in two levels – yes or no). Distinct lowercase letters indicate statistical difference among the experimental groups (all test solutions), within each condition of sucrose exposure). Data were submitted to 2-way ANOVA, followed by Fisher LSD's *post hoc* test for multiple comparisons ($p < 0.05$; $n = 9$). Bars denote standard deviations of the means. Ca: calcium; F: fluoride; P: phosphorus; HMP: sodium hexametaphosphate; CTL: negative control; NANO: nano-sized HMP.

2.4. Determination of P release from HMP

Mean (SD) P release from the solutions at 1, 7, and 30 days of experiment was 10.5 (0.2), 10.9 (0.2), and 51.2 (7.3) $\mu\text{g P/mL}$ for the HMP solutions, respectively, while the corresponding values for the NaH_2PO_4 solutions were 7968.4 (218.9), 7402.9 (364.9), and 7925.0 (156.2) $\mu\text{g P/mL}$, respectively.

3. Discussion

The present study was motivated by the remarkable effects of HMPmicro on the inorganic composition and pH of cariogenic biofilms *in vitro* [14], along with the higher reactivity of HMPnano over micrometric particles [16]. In comparison to HMPmicro, HMPnano had a higher buffering effect on dual-species biofilms of *S. mutans* and *C. albicans*, which was potentiated by F, and increased P levels in both biofilm biomass and biofilm fluid, without reducing F levels in these compartments. On the other hand, HMP significantly reduced Ca levels in both compartments, and exposure to sucrose promoted an overall decrease in the values of all variables analyzed. Thus, the study's null hypothesis was partially rejected.

Repetitive acid challenges resulting from carbohydrate metabolism by cariogenic biofilms lead to consecutive cycles of de- and re-mineralization at the biofilm-tooth interface, further leading to the development of caries lesions [2]. The present study showed that treatment with a solution containing only 1,100 ppm F led to a significantly higher biofilm pH than that observed for the untreated biofilm (*i.e.*, the negative control). This trend is in line with previous data that described the buffering capacity of F on *S. mutans* biofilms. In brief, HF formed at low pH crosses the bacterial cell membrane and dissociates into H^+ and F^- in the cytoplasm, which is more alkaline than the external environment [17]. The intracellular F^- then inhibits the activity of the glycolytic enzymes, leading to a reduction in the acid production from glycolysis. Also, F^- present in the cytoplasm promotes a reduction in the cytoplasmic pH by lowering the glycolytic activity, affecting both acid tolerance and production by *S. mutans* [18, 19].

As for HMP, recent data demonstrated that the exposure to this polyphosphate substantially reduced biofilms' acidogenicity in comparison to untreated biofilms [16] or in comparison to those exposed to F under highly cariogenic conditions [20]. In line with such data, the higher biofilm pH observed for the groups exposed to HMP-containing solutions is possible related to the buffering ability of this phosphate due to its chelating property, according to which HMP in the biofilm fluid leads to the binding of HMP to

H^+ , and of Na^+ to OH^- [21]. The possible formation of HPO_4^{2-} might also have played an important role, given its substantial buffering capacity [14, 21]. Also, these effects were further enhanced by the addition of F, given that combined treatments of HMP and F led to significantly higher biofilm pH in comparison to their counterparts without F, for both concentrations and particle size of the phosphate. In fact, such an additive effect is even more notable when HMP is administered at the highest concentration, since treatments with HMP at 1% combined with F promoted significantly higher pH in comparison to the positive control (*i.e.*, biofilms treated with 1,100 ppm F). Although the actual mechanism of interaction of HMP and F is still unclear, recent data showed that co-administration of the two actives significantly increased the degree of saturation of HF^0 in the biofilm fluid in relation to hydroxyapatite, in comparison to the compounds alone [14].

The reduction of the particle size to the nanoscale was shown to enhance the buffering capacity of HMP at the highest concentration as well, even after exposure to sucrose. This superior effect of nanoparticles over micrometric ones is in line with a recent study using the same dual-species biofilm model, in which the biofilm metabolism, production of biomass, viability of *S. mutans*, and the protein and carbohydrate content from the extracellular matrix were significantly lower for solutions containing HMPnano compared with their micrometric counterparts [16]. This trend can be justified by the higher area/volume ratio and a higher percentage of atoms of nanoparticles, which make them more reactive than conventional ones [22, 23].

Although HMP has a cyclic molecular structure, this study showed that HMP undergoes hydrolysis over time, which helps to explain the dose-response relationship between HMP concentrations in the test solutions and those in the biofilm. Interestingly, the concentrations of F and HMP in the biofilm biomass were significantly lower in groups treated with HMP/HMPnano co-administered with F compared with the actives alone, suggesting that F and P from the treatments might have competed for the same binding sites. The effects above seem to be somehow linked to the superior buffering action of the actives co-administered [14]. The reasons for such a trend, however, remain unclear, demanding further investigations on the topic.

Regarding Ca concentrations, it is noteworthy that biofilms exposed to HMP or HMPnano, regardless of the presence of F, led to negligible Ca concentrations in the biofilm fluid and biomass, which might be the result of the chelating capacity of HMP. It is well documented that this phosphate presents great affinity to cations [24, 25, 26],

which results in the binding of HMP with these elements from the bacterial cell wall. This property provides HMP an antimicrobial activity, what can affect parameters related to the biofilms, such as the microbial viability and metabolism, as well as the expression of the components of the extracellular matrix [16, 27]. The only source of Ca in the present protocol was the AS used as the growth medium, the reason by which the negative control group presented significantly higher concentrations of this ion compared with the other groups. Interestingly, treatments with a solution containing only F led to significantly higher Ca concentrations than the phosphate-containing treatments both in the biofilm fluid and biomass, but lower in comparison to the negative control. Such a trend may be explained by possible interactions between Ca and F, which can have reflected in the biofilm pH as well.

Although the results presented in this work bring relevant information on the activity of HMPnano and F on biofilms, some drawbacks must be emphasized. Firstly, the data on the inorganic composition of the biofilms analyzed were not provided by biofilm-enamel substrate exchanges, what means that the ions evaluated were all from the culture medium (*i.e.*, artificial saliva), so that direct extrapolations of these results to clinical conditions may not be completely appropriate. In addition, despite the two microbial strains (*i.e.*, *S. mutans* and *C. albicans*) used in this study consist of cariogenic-related microorganisms, dental caries is essentially a polymicrobial condition [28], so that future studies using multispecies biofilms could also bring important information on the activity of this nano-phosphate on more complex biofilms.

4. Materials and Methods

4.1. Biofilm growth and treatment of the biofilms with the experimental solutions

C. albicans (ATCC 10231) colonies previously grown 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 (37 °C). *S. mutans* (ATCC 25175) colonies grown on Brain Heart Infusion agar (BHI agar; Difco) were suspended in 10 ml BHI broth (Difco) and statically incubated overnight (5% CO₂ at 37 °C). After 24 h, *S. mutans* and *C. albicans* cells were recovered by centrifugation (at $5,781 \times g$, for 5 min), and washed twice with 10 ml 0.85% NaCl (Sigma-Aldrich, St Louis, MO, USA). Thereafter, the concentrations of *C. albicans* cells were set at 10^7 cells/ml using a Neubauer counting chamber, while *S. mutans* cells were adjusted in a plate reader at 640 nm (EON Spectrophotometer of EON, Biotek, Winooski, VT, USA) to 10^8 cells/ml in

artificial saliva [14, 29]. The dual-species biofilms were grown in 6-well plates (Costar® #3516, Corning Inc., Corning, NY, USA) in artificial saliva supplemented with sucrose (at 5% CO₂, 37 °C), according to the following composition: 1 l demi-water, 4 g sucrose, 2 g yeast extract, 5 g bacteriological peptone, 1 g mucin type III, 0.35 g NaCl, 0.2 g CaCl₂, and 0.2 g KCl; pH 6.8 [12, 25]. All reagents for artificial saliva preparation were purchased from Sigma-Aldrich (St Louis, MO, USA). The growth medium (*i.e.*, artificial saliva) was renewed once daily by removing half of the content of the wells (*i.e.*, 2 ml) and adding the same volume of a fresh artificial saliva [14].

4.2. Determination of the biofilm pH

After the last treatment, the biofilms were gently washed with 1 ml artificial saliva (for 10 seconds), scrapped from the wells with a cell scraper (Kasvi), and transferred to microtubes with the aid of a pipette (MCT-200-C-Axygen). A pH electrode (PHR-146 Micro Combination pH Electrode - Fisher Scientific), previously calibrated with standards pH 7.0 and 4.0, was used for pH determination [14].

In another set of experiments, after the last treatment, artificial saliva was removed from the wells, and the biofilms were exposed to a 20% sucrose solution, for 3 min (as a cariogenic challenge) [14]. The sucrose solution was then removed and the biofilms were scrapped and transferred to microtubes (within 1 min) for pH determination, exactly as described above [14].

4.3. Analysis of the inorganic composition of the biofilm fluid

The microtubes containing the biofilms scrapped were centrifuged (at $15,267 \times g$) at 4° C, for 5 min, and the biofilm fluid was collected [14]. F concentration was analyzed by an ion-selective electrode (Orion 9409 BN) and reference electrode (Orion 900100), both coupled to a potentiometer (Orion – Thermo Scientific). The electrodes were calibrated for F analyses using F standards of 0.09, 0.18, 0.36, 0.72 and 1.44 µg F/ml (for biofilms treated with F-free solutions) and 6.25, 12.5, 25, 50 and 100 µg F/mL (for biofilms treated with F-containing solutions). A total ionic strength adjustment buffer (TISAB II) was used at the same condition as for the samples, at 1:1 ratio [14]. The Ca concentrations were determined by spectrophotometry on a plate reader (EON, Biotek, EUA), at 650 nm wavelength, as described by Vogel [30]. In brief, Arsenazo III was used as colorimetric agent. An aliquot of 5 µL in duplicate for both standards and samples mixed with 50 µL Arsenazo III and 50 µL demi-water was shaken for 60 seconds in a

plate reader prior to obtaining the resulting absorbance. As for P analyses, this was determined according to Fiske & Subbarow [31], while for determining the HMP concentration, this was performed after 1-h water bath heating at 100 °C [14]. For the samples exposed to sucrose, HMP concentration was determined after 6 h water bath heating at 60 °C [14]. The values obtained in millivolt (for F analysis) or absorbance (for Ca, P, and HMP analysis) were converted into µg using the Microsoft Excel software (Version 2010; Microsoft Corp., Redmond, WA, USA), allowing to determine the analytical parameters (linearity, slope, and coefficient of variation).

4.4. Analysis of the inorganic composition of the biofilm biomass

For the evaluation of F, Ca, and P concentrations in the biofilm biomass, 10.0 mg wet weight plaque was weighed on a scale (Shimadzu AUY 220, Kyoto, Japan) in microtubes, and 0.5 mol/ml HCl was added to the microtubes, and further homogenized [32]. The resulting mixture was kept for 3 hours at room temperature under constant shaking (120 rpm), and centrifuged (at $11,000 \times g$), for 1 min. Thereafter, 400 µL of the liquid was removed and 400 µL 0.5 mol/l NaOH was added to the liquid to neutralize it [33].

The F concentration of the biofilm biomass was analyzed as described above for the biofilm fluid, using F standards containing 0.09, 0.18, 0.36, 0.72, and 1.44 µg F/ml (for the biofilms treated with F-free solutions), and 0.8, 1.6, 3.2, 6.4, and 12.8 µg F/ml (for the biofilms treated with F-containing solutions). The concentrations of Ca, P and HMP were determined exactly as described above for the biofilm fluid.

4.5. Determination of P release from HMP

In order to assess the rate of P release from HMP, aqueous solutions containing 1% HMP (test) and 3% NaH₂PO₄ were prepared and kept at 37 °C. The rate of release of free P from the solutions was assessed 1, 7, and 30 days after preparation, according to Fiske & Subbarow [31], as described above.

4.6. Statistical analysis

All experiments were run in biological triplicate, on three different occasions ($n=9$). Data were submitted to 2-way ANOVA, considering exposure to sucrose (in two levels) and study groups (in ten levels) as variation factors. Fisher LSD's test was used as a *post hoc* test for ANOVA to indicate differences between groups (sucrose exposure

within each test solution) and among groups (test solutions within each condition of sucrose exposure). Statistical analysis was conducted using SigmaPlot 12.0 software (San Jose, CA, USA), adopting $p < 0.05$.

5. Conclusions

It can be concluded that (1) HMP at 1% combined with F influenced the pH of the biofilms analyzed, besides affecting most of the inorganic components of the dual-species biofilms, and (2) the reduction of the size of the particles of HMP to the nanoscale led to an enhancement in its buffering capacity. The data presented in this study contribute to the knowledge of how HMPnano and F act on cariogenic-related biofilms and, along with the existing body of evidence, attest the potential use of this association on caries prevention and control.

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Capítulo 3

Effects of sodium hexametaphosphate microparticles or nanoparticles on the growth of saliva-derived microcosm biofilms

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Short title: Sodium hexametaphosphate on microcosm biofilms.

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Effects of sodium hexametaphosphate microparticles or nanoparticles on the growth of saliva-derived microcosm biofilms

Abstract

Objectives: This study evaluated the effects of sodium hexametaphosphate microparticles (HMPmicro) or nanoparticles (HMPnano) on the growth of saliva-derived microcosms biofilms

Materials and Methods: Saliva-derived biofilms were formed on glass coverslips for 24 h. Thereafter, *Streptococcus mutans* (C180-2) was incorporated or not into the biofilms. From that time point onwards, solutions containing 0.2% HMPmicro or HMPnano, combined or not with 220 ppm F, were constantly present in the culture medium. In addition, 220 ppm F alone (220F) and McBain medium without any compound were also tested as positive and negative controls (CTL), respectively. After 96 h, the biofilms were plated on anaerobic blood agar or sucrose agar bacitracin for total and *S. mutans* CFU-counting, respectively. Biofilms' lactic acid production was analysed spectrophotometrically. Data were submitted to ANOVA or Kruskal Wallis' tests, followed by Student Newman Keuls' test ($p < 0.05$; $n = 12$).

Results: HMPmicro or HMPnano led to significantly lower lactic acid production, and significant reductions in total CFU-counting in microcosm biofilms, supplemented or not with *S. mutans*, in comparison to both controls, with significant differences between 220F and CTL. No significant differences were observed among the groups treated with HMPmicro or HMPnano (with or without F). The same trend was seen for *S. mutans* CFU-counting, in biofilms supplemented with *S. mutans*.

Conclusions: HMP significantly reduced total and *S. mutans* CFU counts, as well as lactic acid production by saliva-derived microcosm biofilms.

Clinical significance: These findings in saliva-derived microcosm biofilms suggest that HMP stands as a promising alternative for the control of cariogenic biofilms.

Keywords: Biofilms; Fluorides; Phosphates; Nanotechnology.

Introduction

Dental caries is a chronic polymicrobial disease, characterized by a diet-microbial imbalance, determined by biological, behavioural, psychosocial, and environmental factors, further resulting in progressive dental destruction [1, 2]. Recently, a trend of decline in caries prevalence and incidence worldwide has been observed [3], despite caries still comprises a problematic issue in several communities, especially during childhood [4]. Accordingly, this scenario has encouraged the search for new strategies for caries control, such as the supplementation of fluoridated vehicles (F-vehicles) with different compounds, aiming to increase their preventive/therapeutic action. Among the most commonly studied alternatives, the incorporation of cyclophosphates to F-vehicles has shown to promote marked promising beneficial effects on enamel de- and remineralisation processes [5, 6], on biofilms [7, 8, 9], as well as on the progression of caries lesions in children [10].

Sodium hexametaphosphate (HMP) is one of the most prominent cyclophosphates used due to its remarkable effects both on tooth surfaces [5, 6] and on biofilms [9, 11]. A recent study evaluated the effects of micrometric/conventional HMP (HMPmicro) on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*, demonstrating that this phosphate, combined with fluoride (F), increased the pH and the inorganic composition of the biofilms (*i.e.*, fluoride, calcium, and phosphorus concentrations) [11]. This association was also able to reduce *S. mutans* viability, biofilm metabolism, production of biomass, and the expression of extracellular matrix components (*i.e.*, proteins, carbohydrates, and nucleic acids) [9].

In addition to the above-mentioned to HMPmicro, the use of nano-sized HMP (HMPnano) has also shown promising effects on the improvement of F-vehicles. Recent data from *in vitro* and *in situ* studies showed that the incorporation of HMPnano to a 1,100 ppm F dentifrice promoted a higher effect against enamel demineralisation [12], besides enhancing its remineralising properties in comparison to a conventional dentifrice supplemented or not with HMPmicro [13]. Furthermore, *in situ* data showed that the incorporation of HMPnano to a conventional dentifrice reduced the production of extracellular polysaccharides (EPS) by the dental biofilm in comparison to its counterpart with HMPmicro, and to a conventional dentifrice containing only F [14]. The main rationale for the superior effects of the nanoparticles over the conventional ones is due to properties such as a high ratio of surface area to volume, and a high percentage of atoms

on the surface in comparison to larger particles, which makes nanoparticles more reactive when compared with the micrometric/conventional ones [15].

Considering the above-mentioned effects of HMPmicro on dual-species biofilms, and the promising *in vitro* and *in situ* data on HMPnano in cariogenic models, it would be interesting to investigate whether such effects would also be observed in polymicrobial biofilms using a high throughput active attachment model. This could provide further data on the antibiofilm effects of HMPmicro under a more realistic approach (*i.e.*, active biofilm attachment, and more complex and diverse microbial ecology). Furthermore, in light of the effects of HMPnano on the enamel de- and re-mineralisation processes, and considering the scarce information on the effects of HMPnano on biofilms, the use of a controlled polymicrobial model could also bring useful information on the antibiofilm effects of this nano-polyphosphate.

Thus, this study evaluated the effects of HMPmicro and HMPnano on the growth of saliva-derived microcosm biofilms formed on glass coverslips, in a high throughput active attachment model, so-called *Amsterdam Active Attachment* model (AAA-model) [16]. The study's null hypotheses were that (1) HMP would not affect the formation and the metabolism of the polymicrobial biofilms, and (2) HMP particle size would not influence the above-mentioned variables.

Material and Methods

Processing and characterisation of HMPnano

The processing and characterization of nano-sized HMP were performed according to Dalpasquale et al. [12]. In brief, 70 g of sodium hexametaphosphate ($((\text{NaPO}_3)_6)$, Sigma Aldrich CAS 7785-84-4, UK) was ball-milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of hexane. After 48 h, the material was filtered and sealed with aluminium foil, and the vials were dried at 75 °C (to allow hexane to evaporate). Both micrometric HMP (HMPmicro, not milled) and HMP milled for 48 h (HMPnano) were analysed by scanning electron microscopy (SEM) to evaluate the particle morphology, and by energy-dispersive X-ray spectroscopy to evaluate the particles' crystalline standard. The particles morphology of the compounds consisted of large aggregate particles for HMPmicro (average $2.05 \pm 0.69 \mu\text{m}$) and low-size particles for HMPnano (average $0.38 \pm 0.12 \mu\text{m}$), while the crystalline standard of HMPnano was

similar to the HMPmicro, demonstrating that the nano-synthesis process did not affect the crystallinity of the particles [17].

Saliva collection

The study was approved by the Medical Ethical Committee of the VU University Medical Centre Amsterdam (document number 2011/236). Stimulated saliva was collected from a single subject during gum-base chewing (27 years old, male, caries-free). The donor was required not to brush his teeth for 24 h and not to drink or eat for at least 2 h before salivary donation. The saliva samples were kept on ice during the collection period, being subsequently diluted 2-fold in 60% sterile glycerol (to protect the bacterial cells from cryodamage), aliquoted and immediately stored at -80 °C for further use. The experiments were run with inoculum from the same batch of frozen saliva [16].

Saliva-derived biofilm formation and exposure to the experimental compounds

The biofilms were grown in a high-throughput active attachment biofilm model (AAA-model), assembled with glass coverslips (diameter 12 mm; Menzel, Braunschweig, Germany), as previously detailed by Exterkate et al. [16]. In summary, glass coverslips were attached to a stainless steel lid using nylon clamps. After being assembled, the lid was placed on a stainless steel tray, wrapped in aluminium foil, and autoclaved at 121 °C. The lid was fitted on top of flat-bottomed 24-well plates (Greiner Bio-One, Alphen a/d. Rijn, The Netherlands).

The model was inoculated by adding 50-fold diluted saliva in McBain medium, whose composition for 1.0 L of demi-water consisted of: 2.5 g mucin (M2378 Sigma-Aldrich), 2.0 g Bacto peptone (Difco 0118-01-8), 2.0 g Trypticase peptone (BBL 211921), 1.0 g yeast extract (Bacto 212750), 0.35 g NaCl, 0.2 g KCl, 0.2 g CaCl₂, 0.001 g hemin (Sigma-Aldrich H1652), 0.0002 g vitamin K1, and 50 mmol/l PIPES, pH 7.0 [18].

Saliva-containing McBain medium was added to each well in a 24-well plate (1.5 mL), and the models were then incubated anaerobically (80% N₂, 10% CO₂, 10% H₂) for 8 h, to promote initial attachment of the biofilms to the glass discs; thereafter, the medium was replenished with a fresh McBain medium. After 24 h, the biofilms were exposed to the compounds by adding them to the growth medium (McBain medium) according to the following experimental groups: HMPmicro at 0.2% (HMP), HMPmicro at 0.2% associated with 220 ppm F (HMP/F), HMPnano at 0.2% (NANO), HMPnano at 0.2%

associated with 220 ppm F (NANO/F), and a group exposed only to 220 ppm F (220F), considered as the positive control. The compounds were filter-sterilised to obtain an aseptic condition. The concentrations were adopted to achieve 20% of those used in previous studies and administered as dentifrices [9, 13, 14]. Pure McBain medium was used as negative control (CTL).

To ensure the presence of *S. mutans* representing highly cariogenic conditions, *S. mutans* was incorporated into the biofilm in a different set of experiments. For this condition, *S. mutans* (C180-2) strain was pre-cultured in BHI broth medium (BHI Agar; Difco, Le Pont de Claix, France) for 24 h. Thereafter (on the 2nd day of biofilm formation), 1:50 overnight pre-culture was added to the biofilm.

Twice daily (at 8 a.m. and 4 p.m.) the medium was replenished with fresh McBain medium containing the compounds according to the experimental groups. In addition, the biofilms were grown in unbuffered McBain medium supplemented with 0.2% sucrose (v/v) in the day-period, and in McBain medium containing 50 mmol PIPES as buffer, without the addition of sucrose in the night-period [16], from the 2nd day onwards. This swap of growth conditions during day and night was performed to provide similar conditions for the growth of different groups of microorganisms (*i.e.*, aciduric bacteria or microorganisms which require pH closer to neutral).

Lactic acid production assay

The evaluation of lactic acid production was performed by an enzymatic assay, previously described by van Loveren et al. [19]. In summary, at the end of the biofilm formation period (96 h after the moment of the saliva inoculation), the lid was transferred to a new 24-well plate containing buffered peptone water (BPW) supplemented with 0.2% glucose (1.5 mL/well), and incubated anaerobically (80% N₂, 10% CO₂, 10% H₂) for 3 h at 37 °C to allow acid production. Then, BPW solutions were analysed for lactic acid concentrations using a colorimetric assay. Lactate standards were used for calibration.

Colony-forming units assay

For estimating the number of viable cells, a colony-forming units (CFU) assay was performed. For this, after 96 h, the glass coverslips were withdrawn from the lids and placed into 2 mL phosphate-buffered saline (PBS). Then, the biofilms were dispersed using a sonicator for 2 min at 1 s pulsations at the amplitude of 40 W (Vibra Cell; Sonics&Materials Inc., Newtown, CT), vortexed for 30 s, and the resulting suspension

was serially diluted in cysteine peptone water. These were then plated on tryptic soy agar blood plates for total counts, and on sucrose agar bacitracin (SAB), for *S. mutans*-counting; plates were incubated anaerobically (80% N₂, 10% CO₂, 10% H₂) for 72 h at 37 °C [16]. Bacterial morphology was considered for *S. mutans* counts, given the unselective nature of SAB.

Evaluation of the buffering capacity of the experimental solutions

The buffering capacity of the McBain medium containing or not HMPmicro and HMPnano were evaluated as follows. Unbuffered McBain medium was prepared, and the compounds were incorporated into the medium according to the experimental groups in a final volume of 8 mL. Thereafter, 10 µL of a 3.2% HCl solution was added with a pipette into each solution tube, which was vortexed for 10 s, before pH determination using a pH electrode, previously calibrated with pH 4.0 and 7.0. In total, HCl was dripped five times.

In addition, the pH of the growth spent medium was measured from the second day (in which the experimental compounds were included) to the end of the biofilm formation period. The spent mediums from both “day-periods” (unbuffered McBain medium, supplemented with 0.2% sucrose) and “night-periods” (buffered McBain medium, without the addition of sucrose) growth conditions had their pH determined.

Statistical analysis

Data on the total and *S. mutans* CFU were log₁₀-transformed. Data on total CFU-counting passed normality (Shapiro-Wilk test), and were submitted to 1-way ANOVA, followed by Student-Newman-Keuls’ *post hoc* test. Data on *S. mutans* CFU-counting, as well as on lactic acid evaluation, did not pass normality (Shapiro-Wilk test) and were evaluated by Kruskal-Wallis’ test, followed by Student-Newman-Keuls’ *post-hoc* test. Data on spent medium’s pH passed normality (Shapiro-Wilk test), and were submitted to 2-way, repeated-measures ANOVA, followed by Student-Newman-Keuls’ test. Statistical analyses were performed at a 5% significance level, using the SigmaPlot software (version 12.0; Systat Software Inc., San Jose, USA). The experiments were run in biological triplicates, on four different moments (*n*=12), except for the evaluation of the buffering capacity of the experimental solutions, in which the results were provided from a single experiment.

Results

Treatments with HMP-containing solutions (both micro-sized and nano-sized HMP) led to $\sim 3 \log_{10}$ significantly lower total CFU-counting in comparison to CTL and 220F groups, with significant differences between CTL and 220F, in saliva-derived microcosm biofilms, supplemented or not with *S. mutans*. No significant differences, however, were observed among the groups treated with HMPmicro and HMPnano, regardless of the association with F (Figures 1a and 1b). The same trend above was observed for lactic acid production (Figure 3).

Regarding *S. mutans* counts, similar trends were observed in the saliva-derived biofilms supplemented with *S. mutans*, in which HMPmicro or HMPnano led to significant reductions in comparison to CTL and 220F groups, with significant differences between CTL and 220F (Figure 2). Nonetheless, for the biofilms not supplemented with *S. mutans*, the presence of the bacteria was not observed, given that even for the CTL group, no colony was verified even in the highest biofilm concentration.

In line with the above-mentioned results, for the pH of the spent medium from the “day-period” (*i.e.*, sucrose-containing unbuffered McBain medium), the CTL group presented the lowest pH values, followed by those from the 220F group. Biofilms exposed to HMPmicro and HMPnano, regardless of the presence of F, presented the highest spent medium’s pH (closer to neutral values), without significant differences among them. For the pH of the spent medium from the “night-period” (*i.e.*, buffered McBain medium, without the addition of sucrose), no significant differences were observed among all biofilms, which presented pH close to neutral values (Figure 5). This trend was confirmed in the buffering capacity assay where both HMPmicro- and HMPnano-containing McBain medium, regardless of the presence of F, presented a more sustained pH in comparison to McBain medium containing only F (220F) or nothing (CTL) (Figure 4).

Discussion

Recent data showed that conventional HMP combined with F markedly reduced the viability of *S. mutans* and interfered with several parameters related to a dual-species biofilm of *S. mutans* and *C. albicans* formed in polystyrene well-plates [9, 11]. Despite encouraging, these results cannot be directly extrapolated to more complex polymicrobial consortia, like those found in the oral cavity, which motivated the use of a high-throughput model of biofilm attachment and a polymicrobial saliva-derived microcosm biofilm. The present study demonstrated that HMP interfered with the biofilm analysed,

by reducing the total microbial load and the viability of *S. mutans*, as well as the metabolism of these biofilms, thus leading to the rejection of the first null hypothesis. Given that the aforementioned effects were not influenced by particle size, the second null hypothesis was accepted.

In this study, HMP was shown to markedly interfere with the growth and metabolism of saliva-derived microcosm biofilms actively attached to glass coverslips, especially by reducing the total and *S. mutans* bacterial load, as well as lactic acid production. These findings are in line with recent data from a dual-species biofilm model (*S. mutans* and *C. albicans*), showing that HMP significantly reduced the microbial load of *S. mutans* [9]. This bacterium consists one of the main microorganisms regarded for the onset and development of dental caries, owing to its capacity of metabolizing fermentable carbohydrates, leading to lactic acid production, especially in the presence of sucrose [20, 21]. For the above-mentioned reasons, *S. mutans* has been the target species in anticaries strategies, the reason by which it was incorporated into the salivary inoculum to mimic highly cariogenic conditions and to ensure the presence of this microorganism in the biofilm. Nonetheless, recent studies have thoroughly emphasized the role of the entire microbiome on several diseases, including dental caries [22, 23]. For this reason, although biofilm models including a limited number of microorganisms can provide interesting data in the initial screening, they pose an important limitation regarding extrapolations to real-life conditions.

HMP consists of an inorganic cyclophosphate which, in combination with F, has demonstrated extensive effects on enamel de- and re-mineralization processes, both in the conventional and nano-sized forms [5, 6, 12, 13, 14]. Due to its great affinity to metallic ions such as Mg^{2+} , Ca^{2+} , K^+ , Al^+ , and Fe^{3+} , HMP forms strong complexes with these metals. This leads to HMP's adsorption to the enamel surface and to the retention of charged ions of CaF^+ and Ca^{2+} , by the replacement of Na^+ in the cyclic structure, leading to a reticular HMP formation on tooth surfaces [6, 24, 25]. In addition to these effects on enamel, the affinity to metallic ions seems to play an important role in the interaction between HMP and biofilms as well. Although the mechanism by which this phosphate acts on biofilms is not completely clear, the literature reports that HMP acts by binding to charged ions at the microbial cells' wall, such as Ca^{2+} and Mg^{2+} , which increases cell permeability and, consequently, affects bacterial metabolism [26, 27].

In addition to its chelating properties, HMP's buffering capacity may have also played an important role in the antibiofilm effects observed. When bacteria metabolize

sugars, a decrease in the pH of saliva/medium and biofilm is observed within minutes ($4.5 < \text{pH} < 5.5$), which then slowly begins to rise due to salivary buffers [28]. Interestingly, pH values of the spent medium from the “day-period” (*i.e.*, unbuffered McBain medium supplemented with 0.2% sucrose) were closer to neutral values. Also, HMP-containing solutions were shown to have higher and more sustained buffering capacity compared with the positive control. These trends corroborate those observed for lactic acid production, and are in line with previous data demonstrating that polyphosphate salts present buffering properties on biofilms, even after exposure to sucrose [8, 11, 29]. In addition, it is safe to assume that HMP’s ability to form neutral species such as monohydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate (H_2PO_4^-), contributed to the trends observed in this study [8].

Surprisingly, the effects of HMP_{nano} on the biofilms were not higher than those observed for HMP_{micro}, for none of the variables analysed. Similarly, the effects observed for HMP alone were not enhanced when associated with F, unlike previous studies on enamel de- and re-mineralisation [12, 13, 14]. Although the reasons for such trends are not evident, some factors might help to explain the data obtained. Firstly, unlike previous studies on dental enamel evaluating the effects of the compounds administered as toothpaste slurries twice daily for 1 minute, the present work assessed the effects of the compounds continuously present in the growth medium at 20% of the concentrations of the aforementioned treatments (*i.e.*, 220 ppm F and/or 0.2% HMP) [12, 13, 14]. Thus, it is reasonable to consider that the different modes of administration of the compounds, as well as their concentrations, might have played a relevant role in the present results. Furthermore, it is possible that the duration of exposure to the compounds may have led to a *plateau* effect for all compounds and associations tests, so that any effects of HMP’s particle size and/or the association with F that could be observed during short-term exposure might have not been detected ought to the present exposure model. It is also noteworthy that the antimicrobial pattern reported for *S. mutans* in a dual-species biofilm model was not observed for the fungus *C. albicans*, given that HMP was not able to reduce its viability [9]. It is then reasonable to assume that the polymicrobial nature of the biofilms assessed in this work could have influenced the overall pattern observed. Finally, it should be highlighted that the variables assessed in the studies on enamel de- and re-mineralisation cited above are very different from those studied in the present work so that any direct extrapolation would not be appropriate.

Despite the promising trends on the effects of HMP on polymicrobial biofilms observed in this study, some limitations should be addressed. Considering the interactions between tooth surfaces and biofilms under clinical situations, the lack of a dental substrate does not allow extrapolations of the results observed to conditions involving such interactions (*i.e.*, de- and re-mineralisation). In addition, as above-mentioned, the study model included the constant presence of the compounds in the growth media, which is advantageous as a proof of principle protocol, but does not resemble clinical conditions. In this sense, although the polymicrobial nature of this work adds important information to the body of evidence on the effects of HMP on biofilms, further studies including conditions such as salivary clearance, host-immune influence, and the presence of a mineral substrate may contribute to a better understanding of the actual effects of the compounds tested.

In conclusion, HMP was shown to substantially reduce the total and *S. mutans* loads, as well the metabolism of a saliva-derived microcosm biofilm, regardless of the particle size or the presence of F. The data presented in this work, in line with those previously reported, suggest that HMP is a promising alternative for the control of cariogenic biofilms.

Compliance with ethical standards

Conflict of interests: The authors declare that they have no conflict of interests.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent: Written informed consent was obtained from the participant of the study.

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Figures

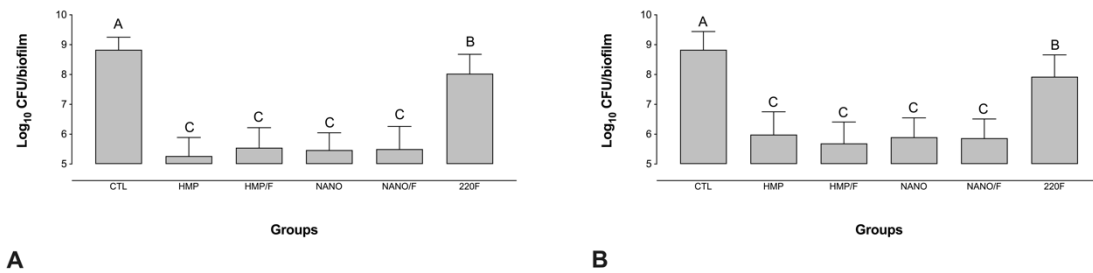


Fig. 1. Log₁₀ total CFU-counting in (A) saliva-derived microcosm biofilms and (B) saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2) according to each experimental group. Bars denote the standard deviations of the means. Distinct letters indicate significant differences among the groups (ANOVA and Student-Newman-Keuls' test, $p < 0.05$, $n = 12$). CTL: negative control (pure McBain medium); HMP: sodium hexametaphosphate; F: fluoride; NANO: nano-sized sodium hexametaphosphate.

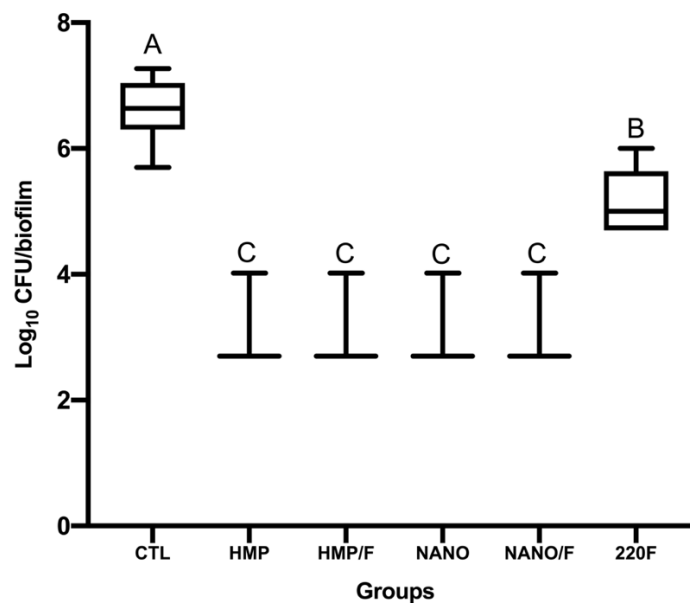


Fig. 2. Log₁₀ *Streptococcus mutans* CFU-counting in saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2) according to each experimental group. Distinct letters indicate significant differences among the groups (Kruskal Wallis' and Student-Newman-Keuls' tests, $p < 0.05$, $n = 12$). CTL: negative control (pure McBain medium); HMP: sodium hexametaphosphate; F: fluoride; NANO: nano-sized sodium hexametaphosphate.

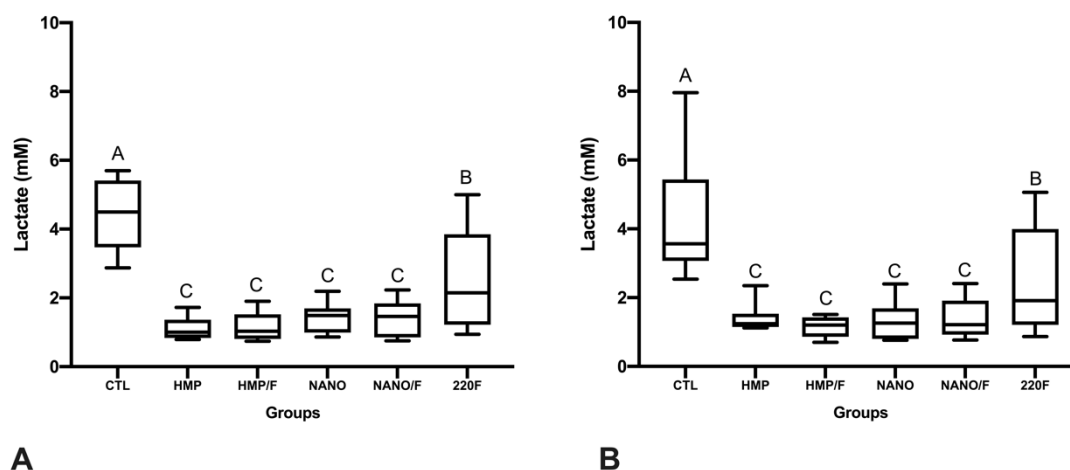


Fig. 3. Concentration of lactate (mM) produced by the (A) saliva-derived microcosm biofilms and (B) saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2) according to each experimental group. Distinct letters indicate significant differences among the groups (Kruskal Wallis' and Student-Newman-Keuls' tests, $p < 0.05$, $n = 12$). CTL: negative control (pure McBain medium); HMP: sodium hexametaphosphate; F: fluoride; NANO: nano-sized sodium hexametaphosphate.

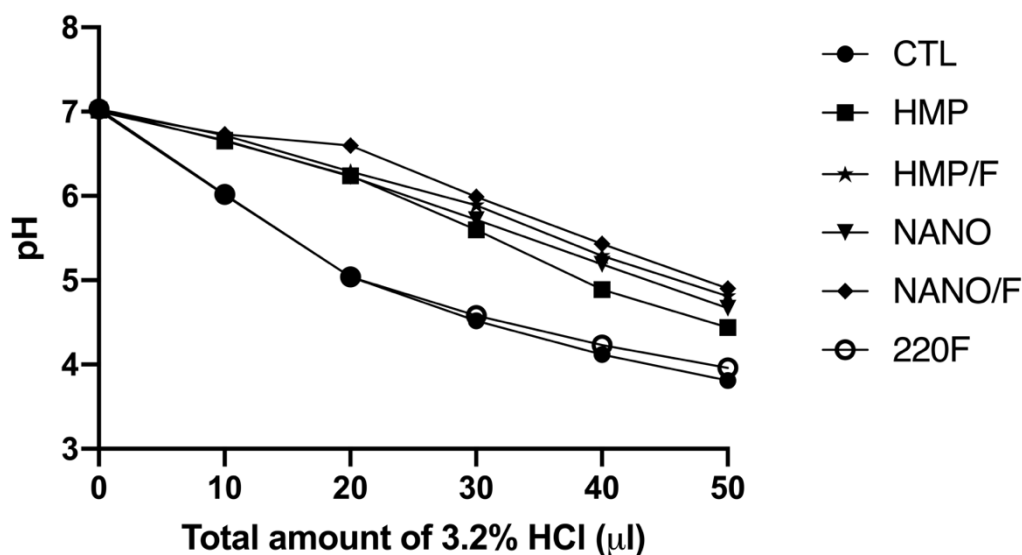


Fig. 4. Buffering capacity of the experimental solutions according to each experimental solution. CTL: negative control (pure McBain medium); HMP: sodium hexametaphosphate; F: fluoride; NANO: nano-sized sodium hexametaphosphate. Results provided from a single experiment.

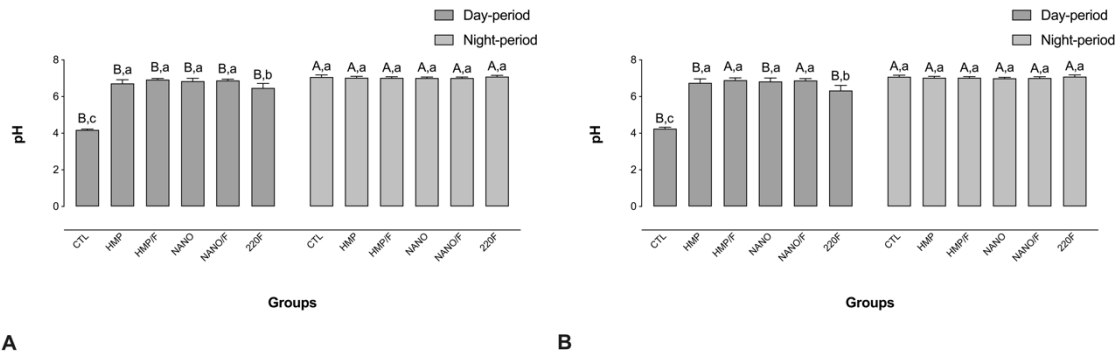


Fig. 5. Mean (SD) pH of the spent medium from the day-period (unbuffered McBain medium supplemented with 0.2% sucrose), and night-period (buffered McBain medium without sucrose) of (A) saliva-derived microcosm biofilms and (B) saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2) according to each experimental group. Distinct upper-case letters indicate significant differences between day and night within each treatment solution. Different lower-case letters represent significant differences among the groups, separately for day and night periods (Two-way, repeated measures ANOVA and Student-Newman-Keuls' tests, $p < 0.05$, $n = 9$). CTL: negative control (pure McBain medium); HMP: sodium hexametaphosphate; F: fluoride; NANO: nano-sized sodium hexametaphosphate.

CONSIDERAÇÕES FINAIS

Em resumo, os dados apresentados acerca dos efeitos do HMPnano sobre a composição microbiana, inorgânica e pH de biofilmes mistos de *S. mutans* e *C. albicans* (Subprojetos 1 e 2) permitem concluir que os tratamentos com soluções combinando HMPnano a 1% com 1100 ppm F levaram a resultados promissores, sendo estes superiores aos obtidos para o controle positivo (*i.e.*, 1100 ppm F) e para as soluções contendo sua contraparte microparticulada (*i.e.*, HMPmicro) combinado com 1100 ppm F. Por fim, observou-se que o HMP promoveu a diminuição da viabilidade microbiana e acidogenicidade de biofilmes microcosmos, sem influência, entretanto, do tamanho da partícula de HMP e da presença de F (Subprojeto 3).

Alguns aspectos importantes inerentes aos desenhos experimentais dos estudos devem ser considerados para a interpretação conjunta dos dados obtidos nos 3 subprojetos. Primeiramente, os estudos realizados com biofilmes mistos de *S. mutans* e *C. albicans* (Subprojetos 1 e 2) incluíam a exposição dos biofilmes aos ativos por meio de tratamentos de curta duração (*i.e.*, 1 minuto), enquanto o estudo realizado com biofilmes microcosmos (Subprojeto 3) envolveu a presença constante dos ativos no meio de cultura, a 20% das concentrações utilizadas nos demais subprojetos. Tal fato acarreta em importantes implicações para a interpretação destes resultados, visto que tratamentos de curta duração são mais equiparáveis às condições clínicas reais em comparação à exposição constante dos biofilmes aos compostos. Por outro lado, a alta complexidade microbiana dos biofilmes microcosmos tem maior proximidade aos biofilmes formados *in vivo* na cavidade bucal, em comparação aos biofilmes de duas espécies.

Sendo assim, tendo em vista os resultados promissores encontrados neste trabalho no que diz respeito à nanopartículas de HMP associadas ao F em biofilmes mistos de *S. mutans* e *C. albicans*, futuros estudos incluindo biofilmes de alta complexidade (como o modelo de microcosmos) deveriam ser desenvolvidos, envolvendo métodos analíticos complementares aos conduzidos neste estudo, e condições de tratamento mais próximas da realidade clínica (*e.g.*, substrato para formação de biofilmes, concentrações dos ativos e modo/tempo de aplicação), a fim de melhor compreender o efeito deste nanocomposto sobre biofilmes cariogênicos.

ANEXO A – REFERÊNCIAS DA INTRODUÇÃO GERAL

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ANEXO B – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA EM SERES HUMANOS

commissie

De Boelelaan 1117
1081 HV Amsterdam

postbus 7057
1007 MB Amsterdam

telefoon 020 444 4444

www.VUmc.nl

dr. R. Exterkate
ACTA/Department of Preventive Dentistry
Gustav Mahlerlaan 3004
1081 LA Amsterdam

Medisch Ethische Toetsingscommissie
VU medisch centrum
voorzitter: Prof. dr. J.A. Rauwerda
intern postadres: BS7, kamer H-565

VU medisch centrum



datum
25 juli 2011

onderwerp
niet WMO advies

ons kenmerk
2011/236

doorkiesnummer
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e-mail subcom-ethiek.org@vumc.nl

Geachte heer Exterkate,

De Medisch Ethische Toetsingscommissie VUmc adviseert in positieve zin omtrent de uitvoering van het onderzoek: **"Onderzoek naar het effect van donoren op de samenstelling en gedrag van biofilms."** Het onderzoek valt niet onder de WMO.

De goedkeuring, waartoe besloten is in het overleg tussen voorzitter en secretaris d.d. 7-7-2011, is gebaseerd op de volgende documenten:

- Protocol, d.d. 23-5-2011
- Patiënteninformatie, d.d. 23-5-2011
- Privacy reglement, d.d. 23-5-2011

Met vriendelijke groet,
namens de METC VUmc,


prof. dr. J.A. Rauwerda, voorzitter


dr. P. de Haan, secretaris

Samenstelling commissie
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dr. K. Hoekman
mw. M. Baak
mw. dr. B. van Baarsen,
dr. M.J.P.A. Janssens en
mw. dr. L.A.M. van der Scheer
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mw. dr. M.A. Bremmer
dr. E.G. Haarman
mw. mr. A.J.G.M. Janssen en
mr. F.J. Faber
dr. D. de Jong

voorzitter, chirurg
plv. voorzitter, internist-oncoloog
verpleegkundige
medisch ethici
biomedicus
psychiater
kinderlongarts
juristen
chirurg

dr. M. Klein
dr. B.W. van Oosten en
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methodologen
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ANEXO C – PROCEDIMENTOS EXPERIMENTAIS

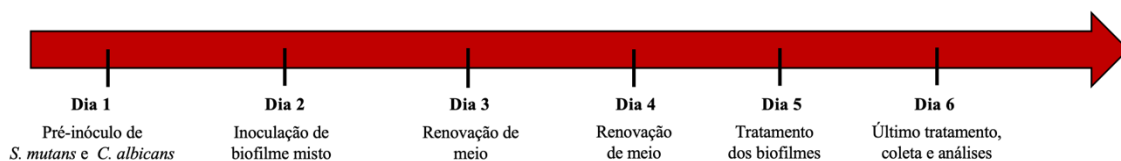


Figura 1. Linha do tempo representando o protocolo de formação e tratamentos dos biofilmes mistos de *S. mutans* e *C. albicans*, bem como coleta e análises. Esquema representativo referente aos capítulos 1 e 2.

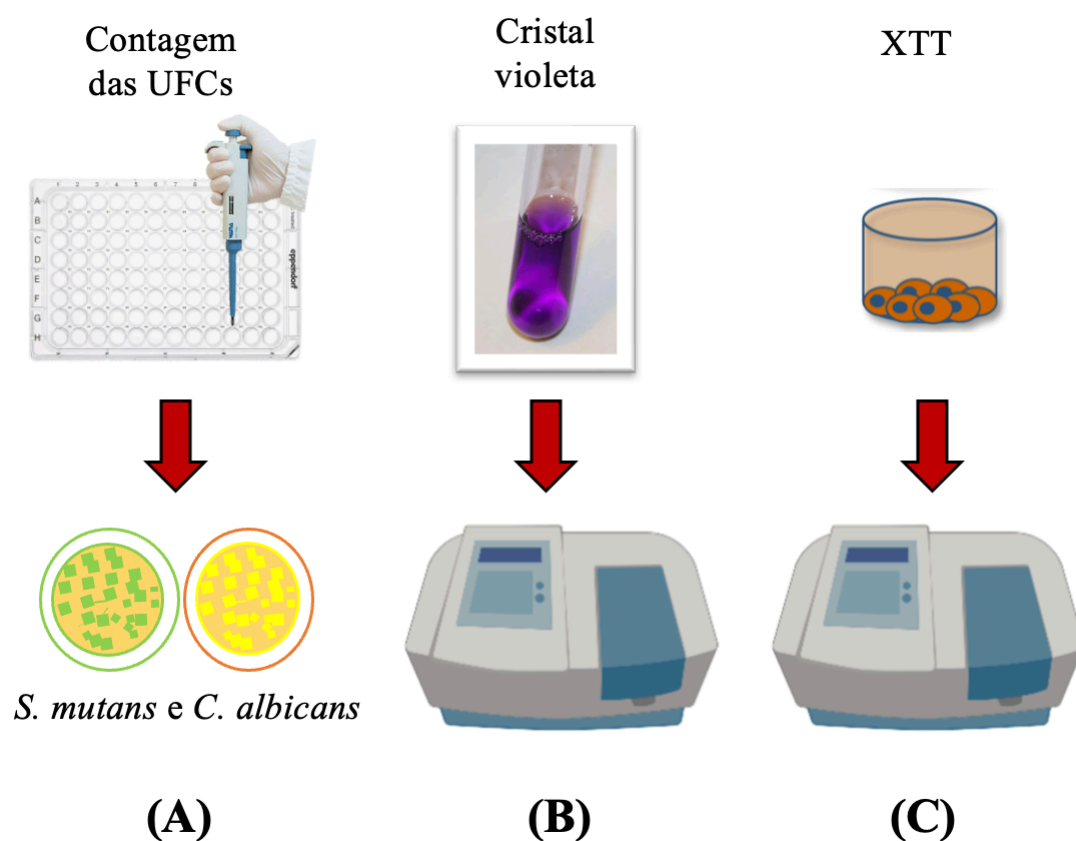


Figura 2. Ilustração esquematizando os protocolos de (A) microdiluição seriada para contagem das unidades formadoras de colônias (UFCs) de *S. mutans* e *C. albicans*; (B) análise da produção de biomassa total (método do cristal violeta); e (C) avaliação da atividade metabólica (método do XTT) de biofilmes mistos de *S. mutans* e *C. albicans*

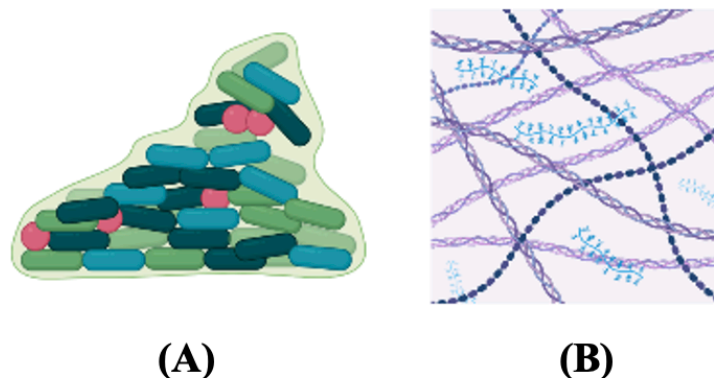


Figura 3. Imagem representativa de (A) biofilme envolto por matriz extracelular; e (B) matriz extracelular e seus componentes (*e.g.*, proteínas, carboidratos). No presente estudo, o conteúdo da matriz extracelular dos biofilmes mistos de *S. mutans* e *C. albicans* em termo de proteínas e carboidratos foram analisados por ensaios colorimétricos (método do ácido bicinconínico e método fenol-sulfúrico, respectivamente).

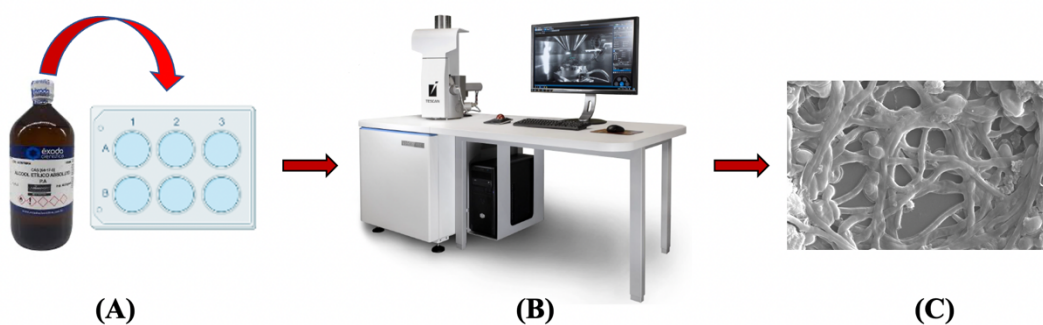


Figura 4. Figura esquematizando a análise estrutural dos biofilmes mistos de *S. mutans* e *C. albicans* por microscopia eletrônica de varredura (MEV). Na figura (A), encontra-se ilustrado o etanol, o qual foi utilizado para fixação dos biofilmes na placa por meio de diferentes diluições, para posterior análise de microscopia.

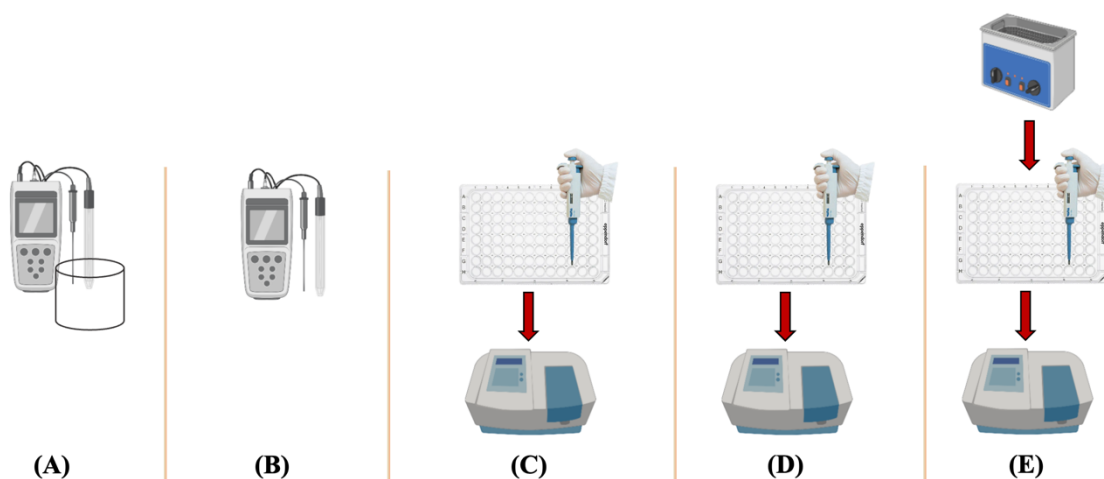


Figura 5. Ilustração esquematizando o protocolo de mensuração de (A) pH, por meio de eletrodo de pH dos biofilmes; e leitura das concentrações de (B) fluoreto, por meio de eletrodo íon-seletivo, (C) cálcio e (D) fósforo, por meio de métodos colorimétricos (por espectrofotometria), e HMP (E), por método colorimétrico (por espectrofotometria), após aquecimento. As dosagens dos referidos íons foram realizadas no fluido e biomassa dos biofilmes.

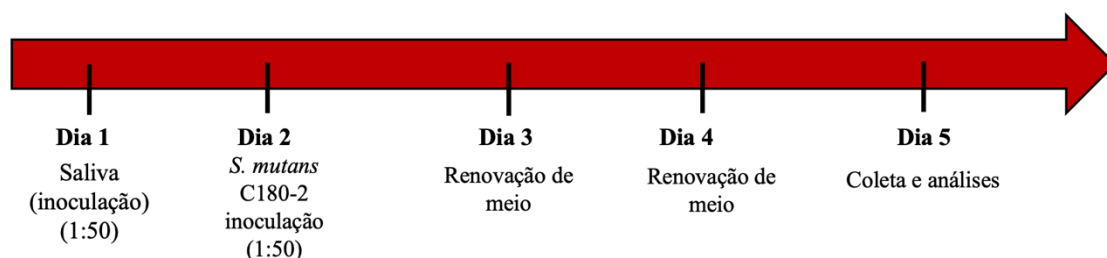


Figura 6. Linha do tempo representando o protocolo de formação de biofilmes microcosmos derivados de saliva, suplementados ou não com *S. mutans*. Esquema representativo referente ao capítulo 3.

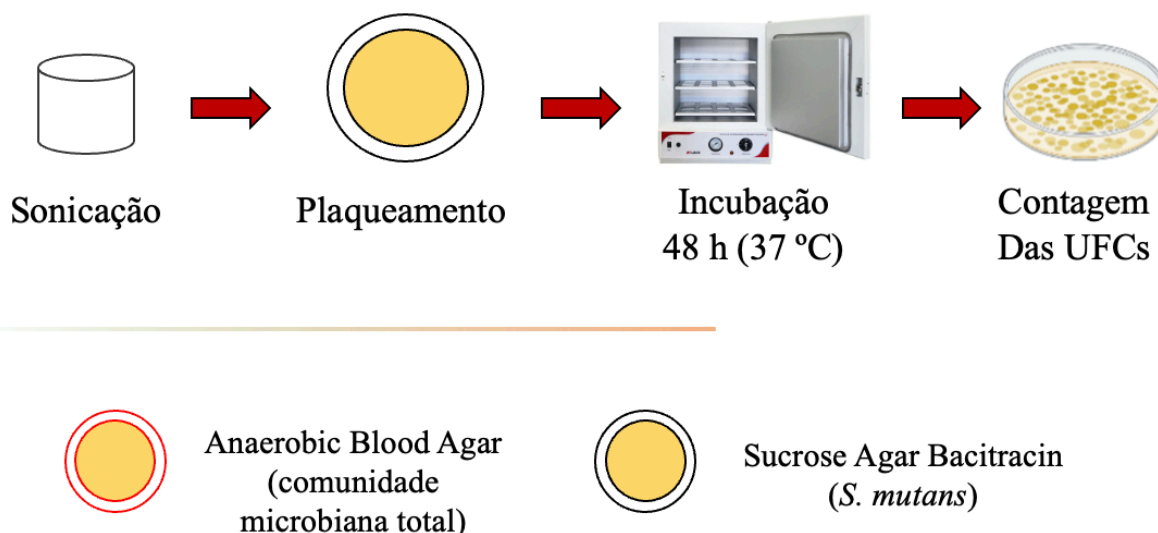


Figura 7 Figura ilustrativa do protocolo para plaqueamento e contagem das unidades formadoras de colônias (UFCs) de comunidade microbiana total e *S. mutans*, em biofilmes microcosmos derivados de saliva, suplementados ou não com *S. mutans*.



Figura 8. Figura ilustrativa do protocolo para avaliação da produção de ácido láctico por biofilmes microcosmos derivados de saliva, suplementados ou não com *S. mutans*.

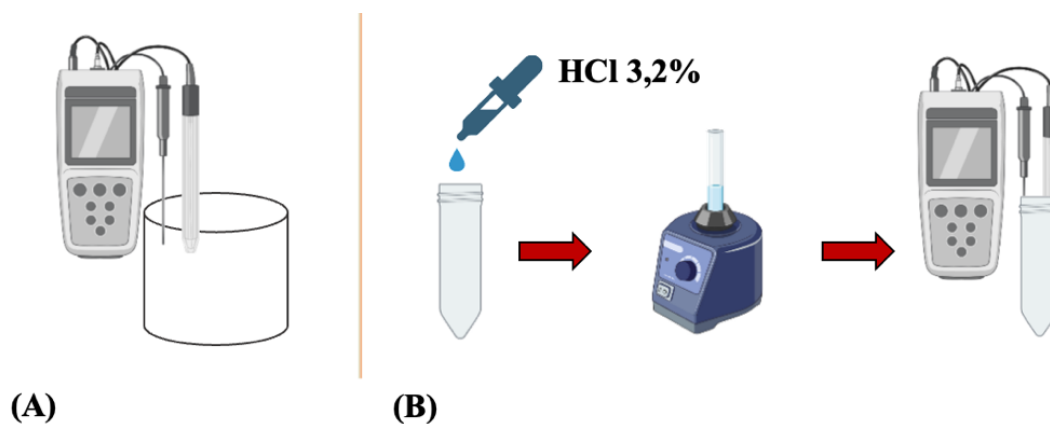


Figura 9. Figura ilustrativa da dosagem de (A) pH do *spent medium* com eletrodo de pH; e esquema da (B) avaliação da capacidade tampão do meio de cultura McBain contendo os ativos estudados, a qual foi realizada por meio da pipetagem de 10 μL de solução de HCl 3,2% por cinco vezes, e mensuração de pH com eletrodo de pH.

ANEXO D – NORMAS DE APRESENTAÇÃO DO PERIÓDICO BIOFOULING

Instructions for authors

Thank you for choosing to submit your paper to us. These instructions will ensure we have everything required so your paper can move through peer review, production and publication smoothly. Please take the time to read and follow them as closely as possible, as doing so will ensure your paper matches the journal's requirements.

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

New nucleic acid sequences must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.

New high throughput sequencing (HTS) datasets (RNA-seq, ChIP-Seq, degradome analysis, ...) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive (SRA).

New microarray data must be deposited either in the GEO or the ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.

New protein sequences obtained by protein sequencing must be submitted to UniProt (submission tool SPIN). Annotated protein structure and its reference sequence must be submitted to RCSB of Protein Data Bank.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "Minimum Information About a Proteomics Experiment". All generated mass spectrometry raw data must be deposited in the appropriate public database such as ProteomeXchange, PRIDE or jPOST. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (<https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/>), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A template permission form is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs' [1]:

Replacement of animals by alternatives wherever possible,

Reduction in number of animals used, and

Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (arriveguidelines.org/) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at <https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf>.

Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: <https://www.animaletics.org.au/three-rs>

Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf

American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: <https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use>

European Animal Research Association. EU regulations on animal research. Available online: <https://www.eara.eu/animal-research-law>.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished de novo cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1+ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country). *Arabidopsis* mutant lines (SALKxxxx, SAILxxxx,...) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (<http://www.consort-statement.org>) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Sex and Gender in Research

We encourage our authors to follow the 'Sex and Gender Equity in Research – SAGER – guidelines' and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full guidelines before submission.

Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved. MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Publication Ethics Statement

Antibiotics is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of Antibiotics take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in Antibiotics must abide to the following:

Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.

Authors should accurately present their research findings and include an objective discussion of the significance of their findings.

Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.

Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.

Simultaneous submission of manuscripts to more than one journal is not tolerated.

The journal accepts exact translations of previously published work. All submissions of translations must conform with our policies on translations.

If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding Updating Published Papers.

Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the Rights and Permissions page.

Plagiarism, data fabrication and image manipulation are not tolerated.

Plagiarism is not acceptable in Antibiotics submissions.

Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.

Image files must not be manipulated or adjusted in any way that could lead to misinterpretation of the information provided by the original image.

Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained. Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work. Authors should not preferentially cite their own or their friends', peers', or institution's publications. Authors should

not cite advertisements or advertorial material. In accordance with COPE guidelines, we expect that “original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations.” This condition also applies to an author’s own work. COPE have produced a discussion document on citation manipulation with recommendations for best practice.

Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Advice on some specific language points can be found here. MDPI provides minor English editing by native English speakers for all accepted papers, included in the APC. The APC does not cover extensive English editing. Your paper could be returned to you at the English editing stage of the publication process if extensive editing is required. You may choose to use a paid language-editing service, such as MDPI’s Author Services, before submitting your paper for publication. If you use an alternative service that provides a confirmation certificate, please send a copy to the Editorial Office. Authors from economically developing countries or nations should consider registration with AuthorAid, a global research community that provides networking, mentoring, resources and training for researchers.

Preprints and Conference Papers

Antibiotics accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal. MDPI operates Preprints, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that Preprints operates independently of the journal and posting a preprint does not affect the peer review process. Check the Preprints instructions for authors for further information. Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. Antibiotics does not publish pilot studies or studies with inadequate statistical power. Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the Proceedings Series journals.

Authorship

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND Drafting the work or revising it critically for important intellectual content; AND Final approval of the version to be published; AND Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE). Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions. For more details about authorship please check MDPI ethics website.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial

submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editorial Independence

Lack of Interference With Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

The suitability of selected reviewers;

Adequacy of reviewer comments and author response;

Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflict of Interests

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.” All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs). Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the MDPI disclosure form. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflict of interest disclosures in the form. See below for examples of disclosures: Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X. If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house Managing Editor to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the Managing Editor will consult the journals’ Editor-in-Chief or Associate Editors to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the Editor-in-Chief.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors’ identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include Editorial Board Members and Guest Editors of the

journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports. Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the Publisher and Editor-in-Chief. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

Accept after Minor Revisions:

The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.

Reconsider after Major Revisions:

The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.

Reject and Encourage Resubmission:

If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.

Reject:

The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an appeal form. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The Managing Editor will forward the manuscript and related information (including the identities of the referees) to a designated Editorial Board Member. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the Editor-in-Chief. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

Promoting Equity, Diversity and Inclusiveness Within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

Resource Identification Initiative

To improve the reproducibility of scientific research, the Resource Identification Initiative aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools. We encourage authors to include unique identifiers - RRIDs- provided by the Resource Identification Portal in the dedicated section of the manuscript. To help authors quickly find the correct identifiers for their materials, there is a single website where all resource types can be found and a 'cite this' button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

ANEXO F – NORMAS DE APRESENTAÇÃO DO PERIÓDICO CLINICAL ORAL INVESTIGATIONS

Instructions for authors

Types of papers

Papers may be submitted for the following sections:

Research Article

Reviews

Brief Report – with up to 2000 words and up to two figures and/or tables

Correspondence (Discussion paper)

Debate (Letter to the Editor)

It is the general policy of this journal not to accept case reports and pilot studies.

Editorial procedure

Clinical Oral Investigations operates a single-blind peer-review system, where the reviewers are aware of the names and affiliations of the authors, but the reviewer reports provided to authors are anonymous.

Submitted manuscripts will generally be reviewed by two or more experts who will be asked to evaluate whether the manuscript is scientifically sound and coherent, whether it duplicates already published work, and whether or not the manuscript is sufficiently clear for publication. The Editors will reach a decision based on these reports and, where necessary, they will consult with members of the Editorial Board.

Summary of the editorial process

The author submits a manuscript and the Editorial Office performs an initial quality check on the manuscript to ensure that the paper is formatted correctly

The manuscript receives a tracking number and Manuscripts are assigned to an Editor-in-Chief or a Section Editor for an initial editorial assessment. If the decision is not to send the manuscript for review, the Editor contacts the author with the decision.

If the Editor decides the paper is within the Journal's remit, peer reviewers are selected and assigned. This can take some time dependent on the responsiveness and availability of the reviewers selected.

Reviewers are given 14 days from acceptance to submit their reports. Once the required reports are submitted, the Associate Editor will give a recommendation or the Editor-in-Chief makes a final decision based on the comments received. The final decision is the sole responsibility of the Editors-in-Chief.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

The title page should include:

The name(s) of the author(s)

A concise and informative title

The affiliation(s) and address(es) of the author(s)

The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

Objectives (stating the main purposes and research question)

Materials and Methods

Results

Conclusions

Clinical Relevance

These headings must appear in the abstract.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text

Text Formatting

Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med.* <https://doi.org/10.1007/s001090000086>

Book

South J, Blass B (2001) *The future of modern genomics*. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. <http://physicsweb.org/articles/news/11/6/16/1>. Accessed 26 June 2007

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