THAMIRES PRISCILA CAVAZANA

Efeito do trimetafosfato de sódio, associado ou não ao fluoreto, na biomassa e fluido do biofilme misto contendo *Streptococcus mutans* e *Candida albicans*

Araçatuba

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Dissertação apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção do título de Mestre em Ciência Odontológica, área de concentração Saúde Bucal da Criança.

Orientador: Prof. Titular Alberto Carlos Botazzo Delbem

Coorientador: Prof. Adjunto Juliano Pelim Pessan, Prof. Dr. Douglas Roberto Monteiro

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RESUMO

O presente estudo teve por objetivo verificar o efeito do trimetafosfato de sódio (TMP), associado ou não ao fluoreto (F), sobre células cultiváveis, biomassa total, atividade metabólica e composição da matriz extracelular de biofilmes mistos de *S. mutans* e *C. albicans*, bem como sobre as concentrações de F, cálcio (Ca) e fósforo (P) (biofilme total e fluido do biofilme) e no pH destes biofilmes formados *in vitro*. Para ambos os estudos, os biofilmes foram formados em poços de placas de microtitulação, colocando uma suspenção ($1x10^7$ células/mL *C. albicans* + $1x10^8$ células/mL *S. mutans*) em saliva artificial suplementada com sacarose (0,4%), a qual tinha metade de seu conteúdo renovada a cada 24 horas. Os biofilmes foram tratados três vezes (72, 78 e 96 horas de formação), por um minuto, com soluções contendo TMP (0,25, 0,5 ou 1%) com ou sem 500 ppm F, além de soluções contendo 500 e 1100 ppm F, adotadas como controles positivos. A saliva artificial foi utilizada como tratamento e considerada como controle negativo. Para o estudo mircrobiológico, após o terceiro tratamento foram realizados os testes de quantificação de células cultiváveis (CFU), biomassa total (teste colorimétrico de cristal violeta – CV), atividade metabólica (redução de XTT) e quantificação dos componentes da matriz extracelular (proteína, carboidrato e DNA). Todos os ensaios foram realizados em triplicata, em três ocasiões diferentes. Os resultados foram submetidos à análise de variância a um critério, seguida pelo teste Fisher LSD ($p<0,05$). O TMP apresentou efeito redutor principalmente no metabolismo e nos componentes da matriz extracelular do biofilme. Para o estudo da concentração de F, Ca, e P, após o período de tratamento, estes foram analisados no biofilme total e no fluido do biofilme após a mensuração do pH do biofilme. Em outro conjunto de experimentos, após o terceiro tratamento (96 h de formação de biofilme) o biofilme foi exposto, por 3 minutos, à solução de sacarose a 20%. Esta foi removida e, após 1 minuto, analisou-se o pH do meio e as concentrações de F, Ca, e P tanto na biomassa como no fluido do biofilme. Os dados foram submetidos a análise de

variância a dois critérios, seguida pelo teste de Fisher LSD ($p<0,05$). O tratamento com TMP aumentou a concentração de F e P no fluido do biofilme, além de manter o pH do meio mais próximo do neutro, mesmo após a exposição do biofilme à sacarose. Assim, é possível concluir que o TMP interfere no metabolismo, composição orgânica e inorgânica, bem como no pH do biofilme testado.

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

Cavazana, TP. Effect of sodium trimetaphosphate, associated or not to fluoride, on biomass and fluid of mixed biofilm containing *Streptococcus mutans* e *Candida albicans*. 2018. 82 f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2018.

ABSTRACT

The aim of the present study was to verify the effect of sodium trimetaphosphate (TMP), associated or not to fluoride (F), on cultivable cells, total biomass, metabolic activity and composition of the extracellular matrix of mixed biofilms of *S. mutans* and *C. albicans*, as well as on the concentrations of F, calcium (Ca) and phosphorus (P) (total biofilm and biofilm fluid) and pH of these biofilms formed *in vitro*. For both studies, the biofilms were formed in wells of microtiter plates by placing a suspension (1×10^7 cells/mL *C. albicans* + $1x10^8$ cells/mL *S. mutans*) in artificial saliva supplemented with sucrose (0,4%), which had half of its content renewed every 24 hours. Biofilms were treated three times (72, 78 and 96 hours of formation), for one minute, with solutions containing TMP (0.25, 0.5 or 1%) with or without 500 ppm F, as well as solutions containing 500 and 1100 ppm F, adopted as positive controls. Artificial saliva was used as treatment and considered as the negative control. For the microbiological study, the following tests were performed: quantification of cultivable cells (CFU), total biomass (colorimetric crystal violet test - CV), metabolic activity (XTT reduction) and quantification of matrix components (protein, carbohydrate and DNA). All assays were performed in triplicate on three different occasions. The results were submitted to one-way analysis of variance, followed by the Fisher LSD's test ($p<0.05$). TMP showed a reducing effect mainly on the metabolism and components of the extracellular matrix of the biofilm. For the study of the concentrations of F, Ca, and P, these ions were analyzed in the total biofilm and in the biofilm fluid after treatment with the test solutions and after the pH measurement of the biofilm. In another set of experiments, after the third treatment (96 h of biofilm formation), the biofilms were exposed for 3 minutes to a 20% sucrose solution. This was removed and after 1 minute the biofilms were collected, and the pH of the medium and F, Ca, and P concentrations were determined both in the biomass and in the biofilm fluid. The data were submitted by two-way analysis of

variance, followed by Fisher LSD's test ($p<0.05$). Treatment with TMP increased F and P concentration of the biofilm fluid, and maintained the pH of the medium close to neutral values even after exposure of the biofilm to sucrose. Thus, it is possible to conclude that TMP interferes with the metabolism, organic and inorganic composition and the pH of the biofilm tested.

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

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LISTA DE ABREVIATURAS

a.m.	Ante Meridiem
Abs	Absorbance
ANOVA	Análise de Variância/Analysis of Variance
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BHI	Brain Heart Infusion
Ca	Calcium
$CaCl_2$	Calcium chloride
CaF_2	Calcium Fluoride
$CaHPO_4^0$	Dicalcium phosphate
CFU	Colony-forming units
cm	centimeter
cm^2	square centimeter
CO_2	Carbon dioxide
CV	Crystal Violet
DNA	Deoxyribonucleic acid
DS	Degree of saturation
EPS	Extracellular polymeric substances
F	Fluoreto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
Fig	Figure
g	Gramme
h	Hour
HA	Hydroxyapatite
HCl	Hydrochloric acid
HF	Hydrofluoric acid
HPO_4^{2-}	Hydrogen phosphate
IA	Ionic Activities
KCl	Potassium Chloride
l	Liter
Log_{10}	Logarithm to the base 10
mg	Milligram

Mg	Magnesium
min	Minute
ml	Milliliter
mmol L^{-1}	Millimolar
mV	Milivolt
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaOH	Sodium hydroxide
NC	Negative Control
nm	Nanometer
p	Probability
P	Phosphorus
p.m.	Post meridiem
pH	Hydrogen potential
ppm	Parts per million
rpm	Revolutions per Minute
s	Second
SD	Standard Deviation
TISAB	Total Ionic Strength Adjustment Buffer
TMP	Sodium Trimetaphosphate
UNESP	Universidade Estadual Paulista
UNOESTE	Univerdidade do Oeste Paulista
XTT	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5Carboxanilide)
µ	Micro
µg	Microgramme
µl	Microliter
°C	Graus Celsius
g	Gravity

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1- Introdução Geral

INTRODUÇÃO GERAL

A cárie dentária é uma doença multifatorial, biofilme-sacarose dependente (Sheiham e James, 2015), ocasionada pela produção bacteriana de ácidos a partir de carboidratos fermentáveis da dieta, a qual progressivamente desmineraliza a estrutura dentária (Cummins e Bowen, 2006). Um dos principais agentes etiológicos da cárie dentária é a bactéria gram positiva *Streptococcus mutans,* devido à sua capacidade de colonizar a superfície dental, metabolizar carboidratos e produzir ácido láctico, além de ter a capacidade de crescer e se multiplicar em ambiente ácido (Marsh e Martin, 2009; Lamont et al., 2006).

O processo de formação de biofilme por esta bactéria inicia-se com o revestimento da superfície do dente pela película adquirida (Bowen e Koo, 2011; Zijnge et al*.,* 2010). A colonização da superfície dentária por *S. mutans* é convencionalmente subdividida em uma fase sacarose independente e uma dependente de sacarose. No biofilme, os microrganismos apresentam-se embebidos em uma matriz extracelular composta por glicoproteínas e polissacarídeos (ten Cate et al., 2009). Inicialmente, várias adesinas de bactérias odontopatogênicas interagem com as glicoproteínas salivares da película adquirida na superfície dos dentes, por meio de ligação a cátions bivalentes. Na presença de sacarose, as bactérias aderem-se firmemente à superfície do dente como resultado da produção de exopolissacáridos (glucanos), por meio da atividade da enzima glicosiltransferase (GTFS). Sendo assim, o acúmulo de biofilme faz com que o *Streptococcus mutans* metabolize eficientemente a sacarose (açúcar ou polímeros, tais como o amido) para produzir grandes quantidades de ácido láctico, capaz de solubilizar o componente mineral do dente e iniciar o processo de cárie (Marsh e Martin, 2009; Lamont et al., 2006).

Além de bactérias, o biofilme dental é composto por vários outros microrganismos, dentre os quais *Candida albicans*, fungo mais comumente encontrado na cavidade oral, que pode ser um fator de risco para o desenvolvimento de cárie dentária (Nikawa et al., 2003). A presença de *C. albicans* é importante na cárie da infância, uma vez que contribui para a patogênese em crianças cárie-ativas (de Carvalho et al., 2006, Klinke et al., 2009). A *Candida* contribui particularmente

para as cavidades dentinárias, por possuir enzimas proteolíticas que realizam a degradação do colágeno (Pereira et al., 2017).

No início do século XXI observou-se um declínio expressivo na prevalência da cárie dentária na maioria dos países desenvolvidos e em desenvolvimento, incluindo o Brasil (Ministério da saúde, Brasil, 2004), sendo que os dentifrícios fluoretados têm contribuído substancialmente para esta redução (Pessan et al., 2011). A utilização dos dentifrícios contendo fluoreto (F) associada à escovação dos dentes é considerada o melhor método preventivo da cárie dentária, visto que associa a remoção ou desorganização periódica do biofilme dental com as propriedades cariostáticas do F (Pessan et al., 2006; Tenuta et al., 2009). Sendo assim, a manutenção da concentração de F na saliva é responsável por seu efeito preventivo e terapêutico, devido ao uso frequente dos dentifrícios. Em acréscimo, a formação de produtos da reação esmalte dentina com F, formando o mineral fluoreto de cálcio (CaF_2), também é responsável pelos efeitos supracitados, uma vez que o depósito destes reservatórios no biofilme dental e em lesões de cárie iniciais é capaz de interferir na progressão da mesma (Buzalaf et al., 2011).

Além do F, as concentrações de fosforo (P) e cálcio (Ca) não somente no biofilme total, mas também no fluido do biofilme, exercem papel fundamental nos processos de des- e remineralização da estrutura dentária. A presença desses íons no ambiente bucal durante o desafio cariogênico proporciona uma diminuição no processo de desmineralização e um aumento no processo de remineralização do esmalte dentário. O processo de remineralização pode ocorrer de duas maneiras: através da precipitação de fosfatos de cálcio ou pelo crescimento dos cristais de esmalte remanescentes através do Ca e P presentes na saliva (Buzalaf et al., 2011). Em acréscimo, a concentração de íons F, Ca e P no biofilme dental apresenta uma relação inversa com a incidência de cárie (Shaw et al., 1983), possivelmente devido à liberação destes íons para o fluido do biofilme, causando uma redução na desmineralização e aumento da remineralização pela supersaturação em relação ao esmalte dentário (Buzalaf et al., 2011).

Concomitantemente ao declínio da carie dentária, tem sido observado um aumento na prevalência de fluorose dentária, sendo a ingestão de dentifrícios fluoretados, especialmente por crianças menores de 6 anos de idade, considerada como o principal fator contribuinte (Warren e Levy, 1999; Mascarenhas, 2000; Wong et al., 2011). Uma alternativa para se minimizar a ocorrência de fluorose é a

diminuição da concentração de F em dentifrícios, embora haja evidência de que tais produtos sejam menos eficazes em crianças com alta atividade de cárie (Lima et al., 2008). Por outro lado, a suplementação com sais de fosfato tem sido uma possibilidade para aumentar a efetividade do F. Estudos *in vitro* e *in situ* demonstraram que dentifrícios com concentração reduzida de F suplementados com trimetafosfato de sódio (TMP) apresentam efeito semelhante à de um dentifrício convencional (1.100 ppm F) sobre a desmineralização do esmalte (Takeshita et al., 2009; Takeshita et al., 2015). Entretanto, os efeitos da associação do TMP e F dependem da proporção entre estes sais (Takeshita et al., 2009; Manarelli et al., 2014) e, assim, para 500 ppm F a concentração ideal de TMP a ser utilizada é de 1% (Takeshita et al., 2011; Takeshita et al., 2015). Em acréscimo, o TMP aumenta significativamente a porcentagem de remineralização de lesões de cárie artificiais (Danelon et al., 2015), uma vez que o efeito deste fosfato está associado à sua capacidade de se adsorver ao esmalte (McGaughey e Stowell, 1977; van Dijk et al., 1980), bem como à retenção de fluoreto de cálcio em suas moléculas previamente aderidas ao esmalte (Danelon et al., 2014; Manarelli et al., 2014). Tais achados foram recentemente confirmados em um estudo clínico randomizado controlado, no qual a progressão de cárie em dentes decíduos foi significativamente menor em crianças que utilizaram um dentifrício contendo 500 ppm F e TMP em comparação a crianças utilizando uma formulação convencional contendo 1100 ppm F (Freire et al., 2016).

Em relação aos efeitos do TMP sobre o biofilme dental, embora a associação entre 500 ppm F e TMP tenha elevado as concentrações de F e Ca no biofilme total a níveis semelhantes aos obtidos quando do uso de um dentifrício convencional (Takeshita et al., 2015), tais aumentos não foram refletidos nas concentrações de F e Ca no fluido do biofilme formado *in situ,* sob desafio cariogênico (Nagata et al., 2017). Com base no exposto, torna-se evidente que enquanto o efeito do TMP sobre o esmalte dentário apresenta grande corpo de evidência científica, os dados sobre os efeitos da associação F-TMP sobre o biofilme dental ainda são escassos e conflitantes. Este aspecto reforça a necessidade de estudos adicionais avaliando os efeitos do F e do TMP sobre o biofilme, especialmente envolvendo métodos analíticos complementares aos utilizados nos estudos supracitados, para uma melhor compreensão dos mecanismos de ação destes íons sobre a dinâmica da cárie dentária.

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

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

- Capítulo 1: **"Activity of sodium trimetaphosphate, associated or not to fluoride, on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*"**
(artigo preparado para a submissão ao periódico Future Microbiology);

- Capítulo 2: **"Effect of sodium trimetaphosphate, associated or not to fluoride, on the composition and pH of mixed biofilms, before and after exposure to sucrose"**
(artigo preparado para submissão ao periódico Caries Research).

**As referências estão no ANEXO A*

2. Capítulo 1

Activity of sodium trimetaphosphate, associated or not to fluoride, on dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

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Este artigo segue as normas da revista Future Microbiology (Anexo B).

Abstract

Objective: To evaluate the effect of sodium trimetaphosphate (TMP), associated or not to fluoride (F), on dual-species biofilm of *Streptococcus mutans* and *Candida albicans*. Materials & Methods: The biofilms of 72 h was treated with 0.25, 0.5 and 1% TMP solutions, combined or not with 500 ppm F, and analyzed by quantification of colony-forming units, metabolic activity, biomass and extracellular matrix components. Results: TMP significantly reduced the number of *S. mutans* cells and biomass only when associated to F. Fluoride-free TMP promoted significant reductions in biofilm metabolism, while all tested solutions decreased the contents of the biofilm matrix compared to untreated groups. Conclusions: TMP alone had a reducing effect mainly on metabolism and extracellular matrix components of the biofilm.

Keywords: Biofilms, *Streptococcus mutans, Candida albicans*, Sodium trimetaphosphate

Introduction

Caries is among the major oral diseases that dental care professionals treat daily in their offices [1]. This disease has multifactorial etiology, being considered sucrose- and biofilm-dependent [2]. *Streptococcus mutans*, a Gram-positive bacterium, is a pathogen frequently found in cases of cavitated caries lesions [3]. However, in view of the polymicrobial nature of the oral biofilm, other species may also contribute to the pathogenesis of dental caries, such as the fungus *Candida albicans* [4]. In fact, *C. albicans* has ability to produce acids and has been significantly associated with early childhood caries [5,6]. In addition, proteolytic enzymes produced by this fungus may degrade collagen, contributing to dentin cavities [7].

In order to prevent dental caries, fluoride (F) products have been widely tested. In this context, the use of F-containing toothpastes showed a positive effect on caries reduction [8], but only for fluoride concentrations of 1000 ppm or above [9]. Thus, the supplementation of low-F toothpastes (~500 ppm F) with phosphate salts has been an alternative to increase F effectiveness. In fact, the clinical efficacy of low-F dentifrices supplemented with sodium trimetaphosphate (TMP) was shown to be higher than that of a conventional dentifrice (1100 ppm F) [10]. *In vitro* and *in situ* studies showed similar effects of a low-F dentifrice combined with TMP and a conventional formulation on enamel demineralization [11, 12]. Furthermore, the remineralizing capacity of a dentifrice with 500 ppm F was improved by the addition of TMP in its composition [13].

Regarding the effects of TMP on dental biofilm, the association between 500 ppm F and TMP was shown to modify the concentrations of F, Ca and extracellular insoluble polysaccharides in the total biofilm at similar levels to those achieved when using a 1100 ppm F dentifrice *in situ* [12]. However, this same trend was not observed for biofilm fluid formed *in situ* under cariogenic challenge [14]. Taking into account the aforementioned context, it is evident that while the effect of TMP on dental enamel presents substantial body of scientific evidence, data on its effects in association or not with F on dental biofilm are still scarce and conflicting.

Thus, the aim of this study was to assess the effect of TMP, associated or not to F, on dual-species biofilm of *S. mutans* and *C. albicans* by different biofilm quantification assays (cultivable cells, total biomass, metabolic activity and extracellular matrix composition).

Materials and methods

Artificial saliva

The culture medium used for biofilm formation was sucrose-containing artificial saliva (AS), and its composition for 1 l of deionized water was based on the protocol recommended by Lamfon et al. (2003) [15], with modifications: 4 g of sucrose (Sigma-Aldrich), 2 g of yeast extract (Sigma-Aldrich), 5 g of bacteriological peptone (Sigma-Aldrich), 1 g of mucin type III (Sigma-Aldrich), 0.35 g of NaCl (Sigma-Aldrich), 0.2 g of $CaCl_2$ (Sigma-Aldrich) and 0.2 g of KCl (Sigma-Aldrich). The pH of the solution was adjusted with NaOH to 6.8.

Preparation of TMP solutions containing or not F

TMP solutions were prepared by weighing and diluting the Trisodium trimetaphosphate salt (Sigma-Aldrich) in appropriate volumes of sterile deionized water to achieve the final concentrations of 0.25, 0.5 and 1%. Next, NaF (Sigma-Aldrich) was added to each TMP solution to achieve a concentration of 500 ppm F. The effects of the combination of TMP with F depend on the proportion of these compounds, and previous studies verified that the lowest ideal concentration of TMP to be used with 500 ppm F was 1% [11, 16]. Solutions with lower concentrations of TMP (0.25 and 0.5%) were used to evaluate possible dose-dependent effects.

Strains and growth conditions

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

Biofilm formation and treatment with TMP associated or not to F

Dual-species biofilms were formed in flat-bottom 96-well microtiter plates (Costar, Tewksbury, USA). For this, 100 µL of each microbial suspension (2 x 10^7 cells/mL for *C. albicans* + 2 x 10^8 cells/mL for *S. mutans*) were added to the wells and the plates, incubated in 5% CO_2 at 37ºC for 72 h. The medium was renewed every 24 h by removing 100 µL and adding an equal volume of fresh AS. Subsequently, 72-h biofilms were treated twice a day (10:00 a.m. and 4:00 p.m.) for 1 min, followed by a further 1-min treatment on the next day (10:00 a.m.) [17], with three different concentrations of TMP associated or not to F: 0.25% (0.25TMP), 0.5% (0.5TMP), 1% (1TMP), 0.25TMP + 500 ppm F (F 0.25TMP), 0.5TMP + 500 ppm F (F 0.5TMP) and 1TMP + 500 ppm F (F 1TMP). After the last treatment, AS was removed from the wells, and the resulting biofilms were washed once with 0.85% NaCl to eliminate planktonic cells. Solutions containing 500 and 1100 ppm F (500F and 1100F) were used as positive controls, whereas AS devoid of TMP and F was considered as negative control (NC).

Biofilm quantification assays

Cultivable cells

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

Total biofilm biomass

Biofilm biomass was quantified by the crystal violet (CV) staining assay, as reported by Monteiro et al. (2011) [19]. Biofilms were fixed for 15 min at room temperature with 99% methanol (Sigma-Aldrich), stained during 5 min with 1% crystal violet (Sigma-Aldrich), and destained by exposure to 33% acetic acid (Sigma-Aldrich). Absorbance values were read at 570 nm and represented as a function of

the area of the wells (absorbance/cm^2). Wells containing AS without microbial cells were used as blanks.

Metabolic activity

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

Analysis of extracellular matrix composition

For this assay, dual-species biofilms were developed in the 6-well plates (Costar) containing 4 mL of the microbial suspension, as described above. After treatment with TMP solutions, biofilms were resuspended in 0.85% NaCl, scraped from the wells, and the liquid phase of the extracellular matrix was extracted by sonication (for 30s at 30 W), as detailed elsewhere [20]. The bicinchoninic acid method (Kit BCA; Sigma-Aldrich) was performed for protein determination of the extracellular matrix, using bovine serum albumin as standard [20], while the carbohydrate content was measured by the well-established method of Dubois et al. (1956) [21], using glucose as standard. For DNA content, a volume of 1.5 µl of the liquid phase of the extracellular matrix was spectrophotometrically analysed (at 260 and 280 nm) in a Nanodrop Spectrophotometer (EONC Spectrophotometer of EONC, Biotek, USA) [22]. Protein, carbohydrate and DNA values were expressed as mg/g of biofilm dry weight.

Statistical analysis

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

Results

Biofilm quantification

Sodium trimetaphosphate without F was not able to significantly reduce the number of *S. mutans* CFUs compared to the NC, regardless of the phosphate concentration ($p > 0.05$; Fig. 1a). However, when TMP was associated to F, significant reductions in the number of CFUs were observed in comparison to the NC ($p < 0.001$; Fig. 1a). In addition, all TMP concentrations combined with F were statistically equivalent to 500 ppm F ($p > 0.05$), showing that the phosphate did not alter the F effect on *S. mutans*. Fluoride concentration also interfered in the results, since 1100 ppm F showed higher reduction (~1.5-$\log_{10}$) in the number of cells than 500 ppm F ($p < 0.001$). For *C. albicans*, no differences were detected among treatments ($p = 0.530$; Fig. 1b).

Treatments with 0.25TMP and F 0.25TMP, promoted significant reductions of 15.8 and 14.0% in the total biofilm biomass, respectively, in comparison to the NC (Fig. 1c). For the other TMP concentrations (0.5 and 1%), only the F-associated groups significantly decreased the biomass compared to the NC, and these phosphate groups did not significantly differ from 500 and 1100 ppm F.

All treatments with F-free TMP significantly reduced the biofilm metabolism when compared to the NC (Fig. 1d). However, an opposite trend occurred for treatments with F-associated TMP, except for the F 0.25TMP group, which also decreased the metabolism. Biofilms treated with all controls had similar metabolic activity (Fig. 1d).

Extracellular matrix composition

All treatments were able to reduce protein, carbohydrate and DNA contents from the biofilm matrix compared to the NC ($p < 0.001$; Table 1). For protein content, F-free TMP at 0.25 and 1% promoted significantly higher reductions than those found for their F counterparts. However, there was no significant difference between the phosphates at 0.5%. The highest reductions in protein content were seen for 1100F and 0.25TMP.

Regarding carbohydrate and DNA contents (Table 1), F-free TMP at all tested concentrations promoted significantly higher reductions than those achieved for their F counterparts. The highest reductions in the content of these components were found for the 0.25TMP and 1TMP groups. Finally, 1100F was more effective in

reducing extracellular matrix components than 500F, except for carbohydrates, in which the effects were statistically similar.

Discussion

The combined use of TMP and F plays a significant role on the demineralization and remineralization of dental enamel [11-13], as previously confirmed by a clinical study that showed lower development of carious lesions in children using low-F dentifrices supplemented with TMP when compared to those brushing their teeth with a conventional formulation (1100 ppm F) [10]. Despite these promising results, new insights on how TMP (associated or not to F) acts in/on biofilms are needed to understand its mechanisms of action, and to develop targeted therapies to control cariogenic biofilms. Thus, these aspects served as stimuli for the development of the current study.

According to the literature, TMP has no effect on the microbiological composition of dental biofilm [23]. This fact was observed in the present study, since TMP alone did not modify the number of *S. mutans* and *C. albicans* CFUs (Fig. 1a and 1b). Nevertheless, solutions containing F-associated TMP reduced the number of *S. mutans* CFUs, and behaved similarly to the 500F solution (Fig. 1a). These findings indicate that the antimicrobial effect on *S. mutans* cells is due to the F action, without interference of the phosphate in this process. In fact, significant reductions in the number of cultivable cells for F-free TMP were not expected, since this compound was tested at subinhibitory concentrations. Minimum inhibitory concentration assays revealed that TMP alone inhibited the growth of *S. mutans* and *C. albicans* in the planktonic state at 6 and 12%, respectively (data not shown). Another interesting finding was that F solutions, combined or not with TMP, had a more pronounced effect on *S. mutans* than on *C. albicans* (Fig. 1a and 1b). Comparing the cellular structure of these microorganisms, *C. albicans* presents more complex and larger cells (yeasts and hyphae) than prokaryotic cells, which might hinder F effects on fungi [18].

As for the quantification of total biofilm biomass, TMP alone also did not affect F performance, since no significant differences between F-associated TMP solutions and their F-free counterparts were observed (Fig. 1c). In addition, the treatment of

dual-species biofilm with 0.25TMP showed the highest decreases in total biofilm biomass and in all extracellular matrix components analyzed (Table 1). Taken together, these findings demonstrate that the effects on biofilm biomass promoted by treatments with all F solutions (with or without TMP) and 0.25TMP are associated with decreases in the number of *S. mutans* cells and disintegration of the extracellular matrix.

It was also noted that TMP alone was able to reduce the metabolic activity of the biofilm cells, regardless of the concentration tested. TMP is considered an inorganic polyphosphate [24] and a chelating agent with ability to bind to certain metal ions [25]. In addition, polyphosphates allow their attachment to essential metals (Ca^{2+} and Mg^{2+}) from the cell wall of Gram-positive bacteria, causing alterations in the bacterial metabolism [26]. As the bioavailability of metal nutrients is a limiting factor for microorganisms [27], possible interactions between TMP and metal ions from both the AS medium and the cell wall of microorganisms might help to explain the reductions in metabolism found for biofilms treated with phosphate. However, when TMP was associated to F, the metabolic activity was equivalent to that found for biofilms treated with solutions containing F at 500 or 1100 ppm, probably due to the effect of F on increasing the metabolic activity, as previously reported [28].

Regarding the biofilm matrix, all treatments displayed an effective disintegration of the extracellular components. In contrast, untreated biofilms showed the highest values of protein, carbohydrate and DNA, probably because they were developed without interferences promoted by any compound. In general, extracellular protein and DNA had a similar reducing pattern among the groups containing F and TMP, which could be a reflection of the effects of these compounds on expression of genes associated with biosynthesis of phosphate and amino acids in the microbial cells [22]. Further, a previous *in situ* study revealed a significant decrease in the concentration of extracellular polymeric substances (EPS) for biofilms exposed to 1100 ppm F toothpaste and low-F dentifrice containing 1% TMP, without differences between the two formulations [12]. As the major components of EPS are glucans [29], the carbohydrate results of the current study are in agreement with the above-mentioned *in situ* study, despite the different models of biofilm formation employed.

Interestingly, the ability of microorganisms to produce extracellular matrix containing water-insoluble glucans is a relevant virulence factor associated with the

pathogenesis of dental caries [30]. This matrix hinders the penetration of drugs or other substances into biofilms and, additionally, may create acidic areas that favor the proliferation of aciduric and acidogenic microorganisms [31], like *S. mutans* and *C. albicans*, favoring the appearance of demineralized areas on the surface of tooth enamel. From a clinical point of view, the reductions observed in Table 1 suggest a great advantage of the evaluated compounds. Consequently, TMP associated or not to F could prevent the formation of acidic niches in biofilms, cell adhesion and aggregation, exchange of genetic information among microorganism, as well as other important functions of the biofilm extracellular matrix [32].

Finally, although the dual-species biofilm model does not represent the polymicrobial nature of the dental biofilm found in the oral cavity, it was necessary to use this model to reduce factors that could interfere in the results. The AS used was supplemented with sucrose because this affects the growth of the biofilm, since it is substrate for the construction of the exopolysaccharide matrix [33]. The data obtained may guide and influence new studies, such as the analysis of molecular effects of TMP on the expression of virulence determinants in cariogenic biofilms.

Conclusions

TMP alone had a reducing effect mainly on the metabolism and on the extracellular matrix components of dual-species biofilm of *S. mutans* and *C. albicans*. The association of TMP with F did not modify the action of fluoride on the biofilm. There is no dose-dependent relationship between the concentration of TMP in the treatment and the effects on the evaluated aspects. These findings bring new perspectives on the mechanisms by which this polyphosphate acts in the control of dental caries.

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Table legend

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

Figure legends

Figure 1. Logarithm of colony-forming units per cm^2 for *S. mutans* (a) and *C. albicans* (b) biofilms, and absorbance values per cm^2 obtained for the total biomass (c) and metabolic activity (d) quantification assays. NC: negative control (untreated biofilms). Error bars denote the standard deviations of the means. Different upper-case letters symbolize statistical differences among the groups ($p < 0,05$).

Table

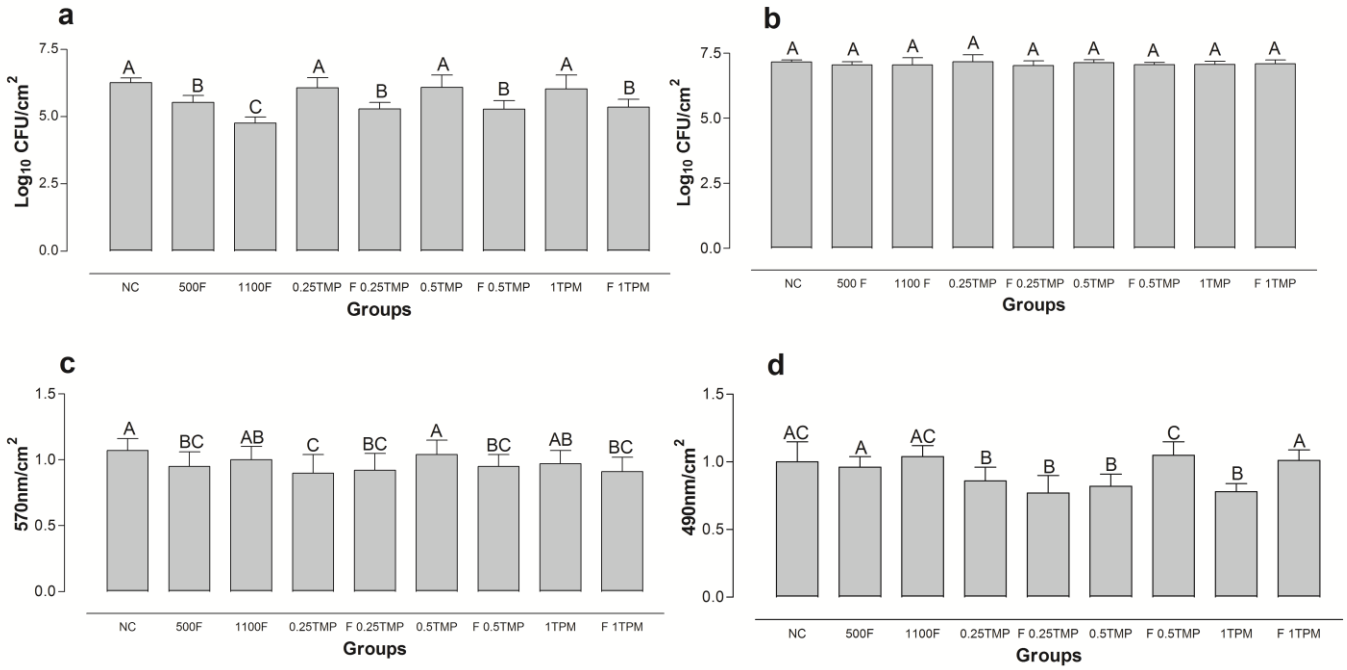
Table 1.

Matrix component (mg/g of biofilm dry weight)	Groups								
	NC	**500 F**	**1100 F**	**0.25TMP**	**F 0.25TMP**	**0.5TMP**	**F 0.5TMP**	**1TMP**	**F 1TMP**
Proteins	25.34^{A} (1.26)	18.93^{B} (1.28)	12.42^{C} (1.69)	12.29^{C} (1.96)	16.80^{D} (1.50)	$17.87^{B,D}$ (1.21)	$16.53^{D,F}$ (1.72)	15.31^{F} (0.42)	20.97^{E} (1.24)
Carbohydrates	542.91^{A} (29.31)	168.17^{B} (16.94)	$160.84^{B,C}$ (15.89)	$149.10^{C,D}$ (15.46)	196.37^{E} (17.16)	173.21^{B} (18.25)	193.15^{E} (14.06)	128.27^{D} (20.84)	158.31^{B} (23.75)
DNA	13.08^{A} (1.97)	9.21^{B} (0.55)	7.85^{C} (0.67)	$7,84^{C}$ (1.43)	10.66^{D} (1.37)	9.57^{B} (0.87)	10.72^{D} (1.29)	6.91^{C} (0.44)	10.71^{D} (2.22)

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

Figure

Figure 1.



3- Capítulo 2

Effect of sodium trimetaphosphate, associated or not to fluoride, on the composition and pH of mixed biofilms, before and after exposure to sucrose

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Running title: Sodium trimetaphosphate in composition and pH of biofilm

Keywords: biofilms, *Streptococcus mutans, Candida albicans*, Sodium trimetaphosphate, Fluorides, Calcium, Phosphate

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***Este artigo segue as normas da revista Caries Research (Anexo C)*.**

Abstract

The aim of the present study was to evaluate the influence of sodium trimetaphosphate (TMP), associated or not with fluoride (F), on the concentrations of F, calcium (Ca) and phosphorous (P), and on the pH of mixed biofilms of *S. mutans* and *C. albicans*, before and after exposure to sucrose. The biofilms received three treatments (72, 78 and 96 hours after the start of their formation), at three TMP concentrations: 0.25, 0.5 or 1%, with or without F at 500 ppm. Solutions containing 500 and 1100 ppm F, and artificial saliva were also tested as controls. Biofilm pH was measured, and the concentrations of F, Ca and P were determined (solid and fluid phases). In a parallel experiment, after the third treatment (96 h) the biofilms were exposed to a 20% sucrose solution, and the pH of the medium, F, Ca, P and TMP were determined. The data were submitted by two-way analysis of variance, followed by Fisher LSD's test ($p<0.05$). Treatment with TMP and 500 ppm F led to higher F concentration in the biofilm fluid. Despite TMP did not affect Ca concentrations, biofilms treated with TMP alone presented higher P concentrations. Treatment with 1% TMP and F led to the highest pH values of the biofilm, both before and after sucrose exposure. It was concluded that TMP increases F and P in the biofilm, and its presence promotes an increase in the pH of the medium, even after exposure to sucrose.

Introduction

Oral acidogenic microorganisms produce the fermentation of carbohydrates, and this process leads to a mineral imbalance between the tooth surfaces and the biofilm formed on them, resulting in the onset of caries lesions [Fejerskov, 2004; Islam et al., 2007]. *Streptococcus mutans* and *Candida albicans* present critical virulence attributes that play an important role in the development of caries [Pereira et al., 2017]. The production of acids by the bacterial metabolism of sugars leads to a decrease in the pH of saliva and biofilm fluid (4,5 <pH <5,5), what renders the medium unsaturated in relation to the dental hydroxyapatite, thus causing its dissolution [Buzalaf et al., 2011]. Fluoride (F), which is widely available in toothpastes ,is known to inhibit the demineralization of hydroxyapatite crystals, and to improve dental remineralization [Featherstone et al., 1999]. Such effects largely depend on the concentration of F in the toothpaste [Wong et al., 2011].

The supplementation of dentifrices with sodium trimetaphosphate (TMP) is an effective alternative to improve the protective effect of F on enamel demineralization, as well as to enhance enamel remineralization [Hirata et al., 2013]. The effect of TMP on low-F dentifrices (500 ppm F) was shown to promote similar F, calcium (Ca) and extracellular insoluble polysaccharides (EPS) of dental biofilm formed *in situ* when compared to those attained by the use of a 1100 ppm F dentifrice [Takeshita et al., 2015]. Surprisingly, such increases were not observed in the concentrations of F and Ca in the biofilm fluid formed *in situ* after a cariogenic challenge [Nagata et al., 2017]. These divergent patterns observed emphasize the need for additional studies evaluating the effects of F and TMP on dental biofilm, especially involving analytical methods complementary to those used in the aforementioned studies, for a better understanding of the mechanisms of action of TMP on these ions in dental caries.

In view of the above, the aim of the present study was to evaluate the influence of TMP, associated or not with F, on the concentrations of F, Ca and P of a mixed biofilm of *S. mutans* and *C. albicans* formed *in vitro* (solid and fluid phases), as well as the pH of this biofilm as a function of sucrose exposure. The null hypothesis was that the presence of TMP does not affect the inorganic composition and the pH of the biofilm, before and after exposure to sucrose.

Materials and methods

Microorganisms and growth conditions

The reference strains used from the American Type Culture Collection (ATCC) *C. albicans* (ATCC 10231) and *Streptococcus mutans* (ATCC 25175) which were kept at -70° C in glycerin. Cultures of *S. mutans* were seeded in Brain Heart Infusion (BHI Agar; Difco) and *C. albicans* in Sabouraud Dextrose Agar (SDA, Difco, Le Pont de Claix, France). *S. mutans* plates were maintained in 5% CO_2 at 37° C for 24 hours, and plates of *C. albicans* were incubated for 24 hours at 37° C. After growth in agar, colonies of *S. mutans* was suspended in 10 mL of BHI broth (Difco) and incubated statically overnight in 5% CO_2 at 37° C. *C. albicans* was suspended in 10 ml of Sabouraud Dextrose broth (Difco) and incubated at 37° C overnight under shaking at 120 rpm [Monteiro et al., 2013]. The cells were then recovered by centrifugation (8000 rpm, 5 min) and washed twice with 10 ml of saline (0.85% NaCl). Using a Neubauer Chamber, the fungal cells were adjusted to a concentration of 10^7 cells/mL in artificial saliva [Fernandes et al., 2016]. The amount of bacterial cells were adjusted spectrophotometrically (640 nm) at a concentration of 10^8 cells/mL of saline (0.85% NaCl) [Fernandes et al., 2016]. The artificial saliva used [Lamfon et al., 2003] was supplemented with sucrose and thus had the following composition for 1 L of deionized water: 2 g of yeast extract (Sigma-Aldrich, St Louis, USA), 5 g of bacteriological peptone (Sigma-Aldrich), 4 g of sucrose (Sigma-Aldrich), 1 g of mucin (Sigma-Aldrich), 0.35 g NaCl (Sigma-Aldrich), 0.2 g $CaCl_2$ (Sigma-Aldrich) and 0.2 g KCl (Sigma-Aldrich). The pH of the saliva was adjusted with NaOH to 6.8. To form the biofilms, 4 mL suspension (1×10^7 cells/mL *C. albicans* + 1×10^8 cells/mL *S. mutans*) in artificial saliva were added to the wells of 6-well microtiter plates (Costar - Coring, NY). These were incubated at 37° C for 72 hours and every 24 hours 2 mL of the artificial saliva was renewed.

Treatment of biofilms and pH measurement

The biofilms were treated 3 times (72, 78 and 96 hours after the start of their formation) for 1 minute [Koo et al., 2005] with solutions containing three different concentrations of TMP: 0.25, 0.5 and 1%, with or without 500 ppm F (0.25% TMP/F, 0.5% TMP/F, 1% TMP/F, 0.25% TMP, 0.5% TMP, 1% TMP, respectively). As

controls, solutions containing 500 and 1100 ppm F, and artificial saliva without F or TMP were used. The biofilms were gently washed with 1 mL of artificial saliva for 10 seconds after the third treatment. Following, biofilms were scraped with a cell scraper (Kasvi) and transferred, with the assist of a pipette, to microtubes (MCT-200-C-Axygen), for pH determination using a pH electrode (PHR-146 Micro Combination pH Electrode - Fisher Scientific), previously calibrated with standards with pH 7.0 and 4.0. The experiments were performed in triplicate, on three different occasions.

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

Analysis of F, Ca and P in the biofilm fluid

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

Calcium was measured by spectrophotometry on a plate reader (EONC Spectrophotometer of EONC, Biotek, USA) at wave length of 650 nm, adapting the method described by Vogel [1983]. In brief, Arsenazo III was used as a colorimetric reagent. An aliquot of 5 µL in duplicate for both standards and samples was mixed with 50 µL of Arsenazo III and 50 µL of deionized water. They were then agitated for 60 seconds in the microplate reader, promoting the reaction between the sample and Arsenazo III before obtaining the resulting absorbances.

Total phosphorus was measured according to the method of Fiske and Subbarow [1925]. The determination of P from TMP was performed using the protocol proposed by Anderson et al. [1977]. For the samples that were exposed to sucrose, the

determination of P from TMP, the boiling-water bath process was replaced by storage of the solutions at 60° C for 6 hours.

Analysis of F, Ca and P in the biofilm biomass

For the analysis of the inorganic composition of the biofilm biomass, 0.5 mol/L HCl was added to the microtubes containing the biofilms at the proportion of 0.5 mL/10.0 mg plaque wet weight [Cury et al., 2000], and homogenised. The resulting mixture was kept for 3 hours at room temperature and under constant stirring (120 rpm), and then centrifuged (11,000 × *g*) for 1 minute [Nobre Dos Santos et al., 2002]. A known amount of the liquid was removed and the same volume of 0.5 mol/L NaOH was added.

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

Determination of ionic activities and degree of saturation from the biofilm fluid

The ionic activities (IA) of species involved in enamel remineralization ($CaHPO_4^0$, HPO_4^{2-}, CaF^+ and HF^0) were calculated from the concentrations (mmol/L) of calcium, fluoride, and phosphorus in the biofilm fluid of each group [Cochrane et al., 2008]. Also, degree of saturation (DS) of the solid phases of hydroxyapatite (HA) and calcium fluoride (CaF_2) were determined. All calculations were performed for conditions at 37° C, pH and density of 1.0 g/cm^3 by the PHREEQC Interactivef (version 2.18.3, U.S. Geological Survey Branch of Information Services, Denver, CO, USA) speciation program. The pH values determined at the end of the experiments for each group were used for the calculation of IA and DS.

Statistical analysis

Data were analyzed by using the statistical program SigmaPlot version 12.0 (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). Shapiro-Wilk's test was used to verify the normality of the data. Two-way analysis of variance was

performed, followed by Fisher LSD's test. All tests were performed with a significance level of 5%.

Results

F, Ca and P in the biofilm fluid

The concentrations of F, Ca and P ions and TMP in the biofilm fluid were lower after exposure to sucrose when compared with those from biofilms not exposed ($p<0.001$), regardless of the treatment (Figure 1). For fluoride concentrations, a direct dose-response relationship was observed among negative control, 500 and 1100 ppm F groups (Figure 1A), while an inverse dose-response relationship was seen for calcium data (Figure 1B). The association F/TMP led to higher fluoride levels when compared to 500 ppm F group, but lower than the 1100 ppm F for biofilms not exposed to sucrose ($p<0.001$). Calcium concentrations were not significantly different among groups treated with TMP ($p>0.112$), achieving similar values seen for the 500 ppm F group ($p>0.169$). After exposure to sucrose, calcium levels in biofilms treated with TMP were similar to those obtained for 500 ppm F ($p>0.093$) and 1100 ppm F ($p>0.168$) groups. P concentrations were higher for groups treated with TMP without F, both before and after exposure to sucrose ($p<0.001$; Figure 1C), when compared to the others groups. F/TMP groups showed similar values of P ($p>0.116$) and no difference compared to the negative control group ($p>0.175$). The amount of TMP in the biofilm fluid (Figure 1D) was higher when TMP was associated with F ($p<0.009$). A dose-response relationship was observed for TMP concentrations in the biofilm fluid and in the treatment solutions.

F, Ca and P in the biofilm biomass

The concentrations of F, Ca and P ions and TMP in the biofilm biomass were lower after exposure to sucrose when compared with those from biofilms not exposed ($p<0.001$), regardless of the treatment (Figure 2). Regarding fluoride (Figure 2A) and calcium (Figure 2B) concentrations, a direct dose-response relationship was observed among negative control (artificial saliva), 500 ppm F and 1100 ppm F groups. The F/TMP groups showed significantly lower F values in biofilms not exposed to sucrose ($p<0.001$) when compared to 500 ppm F, while similar values

among these groups were observed after exposure to sucrose ($p>0.091$). As for Ca concentrations, values of the negative control group were similar to those of biofilms treated with TMP, with or without F ($p>0.624$). Also, similar Ca values were observed for the group 500 ppm F and those treated with TMP/F ($p>0.076$) for biofilms not exposed to sucrose. After exposure to sucrose, Ca concentrations were similar between the negative control group and those treated with TMP solutions ($p>0.069$). With respect to P in the biomass (Figure 2C), significantly higher values were observed for biofilms treated with TMP only, showing a dose-dependent pattern. However, P concentrations were lower when the TMP was associated with F ($p<0.001$). After exposure to sucrose, only the 500 and 1100 ppm F groups showed no significant difference in P concentration when compared to biofilms not exposed to sucrose ($p>0.381$ and $p>0.709$, respectively). As for TMP levels in the biomass, these were proportional to those seen in the treatment solutions (Figure 2D); 1% TMP/F promoted higher amount of TMP in the biomass than the 1% TMP ($p<0.001$). After sucrose exposure, however, a reduction in TMP values was observed when compared to biofilms not exposed to sucrose ($p<0.036$). TMP/F groups showed higher amount -of TMP when compared to TMP-only groups.

pH measurement

The pH of the biofilms after exposure to sucrose was lower when compared with those not exposed ($p<0.001$), except 0.5% and 1% TMP groups (Table 1). A direct dose-response relationship was observed among negative control, 500 ppm F and 1100 ppm F groups and the pH of the biofilm. Treatment with 1% TMP and F led to the highest pH compared to all other groups, being 10% greater than 1100 ppm F, regardless of the exposure to sucrose (Table 1). The pH of biofilms treated with TMP at 0.25 and 0.5% with 500 ppm F did not significantly differ from that in the biofilm treated with 1100 ppm F.

Determination of ionic activities and degree of saturation from the biofilm fluid

The degree of saturation in relation to hydroxyapatite prior to sucrose exposure (Table 2) was higher in the groups treated with TMP and F compared to the other

treatments, among which 1% TMP/F attained the highest values. The lowest saturation was observed in the 1100 ppm F group. After exposure to sucrose, the degree of saturation decreased for all groups. As for the saturation in relation to CaF_2 (Table 2), the highest values were also found for groups treated with TMP and F, but without significant differences among TMP concentrations. The lowest degrees of saturation were observed for groups treated with TMP alone. The 1100 ppm F presented a lower degree of saturation than 500 ppm.

The possible formation of $CaHPO_4^0$ and HPO_4^{2-} (Figure 3A and B) showed a dose-response relationship with TMP concentrations in the treatment solutions; higher amounts of these species were observed when TMP and F were co-administered, except for TMP at 1%. Treatment with 1100 ppm F reduced the formation of $CaHPO_4^0$ compared to the 500 ppm F group, and this relationship was inverse to that seen for the formation of HPO_4^{2-}. The highest CaF^+ formation (Figure 3C) were observed in the groups receiving 500 ppm F, with and without TMP, and were higher for TMP solutions at 0.5 and 1%. The lowest formation was observed for the group of 1100 ppm F. The possible formation of HF^0 (Figure 3D) was higher in the groups that presented F in the treatment solution, after sucrose exposure.

Discussion

The present study aimed to assess the influence of TMP and F, alone or in association, on the inorganic composition and pH of a mixed biofilm *in vitro.* Treatment with TMP and fluoride led to a significant increase of F concentrations in the biofilm fluid, while treatments with TMP alone significantly increased P levels in the fluid and biomass of the biofilms. The pH was also influenced by treatment with TMP and F, being higher when the biofilm received TMP and F in association. However, TMP did not affect Ca concentrations in the biofilm. Thus, the null hypothesis of this study was partially rejected.

The pH of the biofilm was associated to the acid production of the microorganisms after exposure to sucrose, and was shown to be influenced by F and TMP in the treatment solutions. In relation to F, a dose-response relationship was observed between fluoride concentration in the treatment solutions and the pH of the biofilm, as reported in other studies with different protocols [Pandit et al., 2011;

Pandit et al., 2013]. An *in vitro* study reported a decrease in the acidogenicity of *S. mutans* promoted by F, as well as inhibition of the synthesis of extracellular polysaccharides by decreasing the genetic expression linked to glucosyltransferases and to glycolysis, which are processes that lead to pH fall [Pandit et al., 2017]. In addition, the formation of HPO_4^{2-} (Figure 3B) reflects on the pH (Table 1) determined before and after sucrose exposure, since mono-hydrogen phosphate has a buffering effect [Aranha, 2002]. The biofilm treated with 1100 ppm F had ionic composition in the biofilm fluid that favored the formation of this compound, which may have helped in the neutralization of the biofilm observed prior to exposure to sucrose. Other groups that might have formed larger amounts of HPO_4^{2-} contained F and TMP in the treatment solutions (figure 3B), which may also have reflected on the pH of these groups (the highest values observed among all groups). Other phosphate with buffering capacity is di-hydrogen phosphate ($H_2PO_4^-$) and the groups with pH lower than 6.8 presented higher values when compared to the values of HPO_4^{2-} (data not described). Furthermore, the high concentrations of TMP in the fluid phase of the biofilm might also have interfered with the resulting pH, since this is an inorganic polyphosphate used as a buffering agent [Lanigan, 2001]. In addition, treatments with TMP and F presented higher concentrations of both phosphate and fluoride in the biofilm fluid, which consequently affected the pH.

The effects of TMP and F when co-administered on the pH and on F concentrations in the biofilm fluid leads to a higher degree of saturation in relation to HA and CaF_2, affecting favorably the dynamics of de- and re-mineralization of dental enamel [Buzalaf et al., 2011]. In addition, these solutions promoted higher TMP concentrations in the biofilm fluid, and this greater bioavailability is likely to enhance the adsorption of this phosphate to dental surfaces, what may also impact on the mineral dynamics of them. All the above taken together provide new information on how TMP reduces enamel demineralization *in situ* [Takeshita et al., 2015] and caries progression in a clinical trial [Freire et al., 2016].

Considering the formation of CaF^+, it was higher in groups that presented higher concentrations of F and/or Ca. Treatment with TMP and F also increased the formation of CaF^+, possibly due to the high amount of F in the biofilm fluid, since TMP did not impact Ca concentrations in the fluid. While a similar trend had already been described in the fluid of biofilms formed *in situ* [Nagata et al., 2017], TMP was

shown to enhance Ca uptake by the biomass *in situ* [Takeshita et al., 2015]. Such differences may be related to the effects of saliva *in situ*, which, among other functions, regulate the ionic activity in the environment [Mandel, 1987]. Furthermore, TMP is considered an inorganic polyphosphate [Lanigan, 2001] and, therefore, a possible metal chelator [Lee et al., 1994], which may increase Ca concentration in the biomass (from saliva) under *in situ* conditions, differently from the *in vivo* model presented.

Ca levels in the biofilm fluid also influenced its saturation in relation to the HA. Group treated with 1100 ppm F presented the lowest saturation probably due to the lower amount of Ca, which is one of the components of HA. The lower Ca levels also justify the lower saturation of the fluid with respect to CaF_2, and the lower possibility of $CaHPO_4^0$ and CaF^+ formation. Moreover, treatment with 1100 ppm F promoted higher amounts of Ca in the biomass, and the concentrations of F and Ca were proportional, in line with previous data showing that biofilm F concentrations are dependent on Ca levels in the biomass [Pessan et al., 2006]. It is known that cations in the biofilm can induce F binding to bacterial cells [Domon-Tawaraya et al., 2013]. For bivalent cations (such as Ca), the bidentate bonding to the bacterial wall becomes monodentate in the presence of F, as this ion competes with macromolecular anionic groups. This leads to the binding of F to bacteria by a Ca bridge (Ca-F) and increases the negative sites in bacteria, what in turn favors further cation bonding to the bacteria surfaces [Rose et al., 1996].

The fact that TMP can act as a metal chelator may also influence the interaction between *S. mutans*, Ca and F. It is possible that Ca sequestration by TMP decreases the number of F bonds on the bacteria, decreasing F levels in the biomass, thus releasing F ions to the fluid phase. The results of F concentrations in the solid and fluid phases of the biofilms confirm the above-mentioned hypothesis. The chelating capacity of TMP may also be related with increases of P in the biofilm when TMP was administered alone (*i.e.,* without F). Interestingly, yeasts are able to develop mechanisms of nutrient absorption if they are in a deprived medium, as in the case of Mg sequestration [Klompmaker et al., 2017]. These microorganisms can perform endocytosis and capture the nutrient with the chelating molecule, and then, among other mechanisms, destroy the molecule and return parts to the medium [Klompmaker et al., 2017]. Following this rationale, it is possible that TMP, when

alone in the treatment solution, led to higher chelation of metals in the medium; subsequently, *C. albicans* may have captured the TMP-metal complexes, and then released the elements of the TMP molecule, increasing the P of the biofilm, and decreasing the amount of TMP, as observed in the present results.

The increased P availability in the biofilm fluid after treatment with TMP-containing solutions may have interfered in the formation of $CaHPO_4^0$ (Figure 3A). It is hypothesized that TMP administered with F releases CaF^+ species to saliva, which can react with H_2PO_4 forming $CaHPO_4^0$ and HF^0. The results observed in this study support this hypothesis since the compounds mentioned are in greater proportion in the biofilm fluid that received TMP and F. It has been reported that the formation of neutral $CaHPO_4^0$ is of paramount importance when considering enamel remineralization, since the diffusion coefficient of this compound into subsurface lesions is a thousand times greater when compared with ionic calcium [Cochrane et al., 2008].

The concentrations of F, Ca and P of the biofilm of the present study were reduced after exposure to sucrose, as observed *in situ* [Cury et al., 1997] and *in vivo* [Bayrak et al., 2011]. It is already known that almost all biofilm minerals suffer a reduction after the cariogenic challenge [Tenuta et al., 2006]. Exposure to sucrose also led to a reduction in the degree of saturation with respect to HA and CaF_2, as F, Ca and P levels also decreased. With the pH drop caused by the metabolism of carbohydrates, F released to the biofilm fluid leads to HF^0 formation, which penetrate through cell walls and bacterial membranes. After HF^0 ionizes in the cytoplasm, H^+ and F^- are released, and F^- will act on the enzyme enolase (which affects the lactic acid formation pathway) and on ATPases (inhibiting the exit of protons from the cell) [Jenkins, 1999]. This will decrease acid production and limit pH in biofilms [Marquis et al., 2003]. Furthermore, higher quantities of HF^0 were observed after sucrose exposure, what is in agreement with the increase in the formation of this compound. In other groups, the amount of HF^0 decreased after exposure to sucrose, probably due to the penetration of this molecule into the cell of the microorganism, which is facilitated under acidic conditions. For the groups in which the amount of HF^0 was not affected by sucrose, it could reflect a balance between formation of this species and its penetration into the bacterium.

The combination of 1% TMP and 500 ppm F significantly alters the inorganic composition of the biofilm, which may affect the caries dynamics. An important aspect is that this combination is able to resist more efficiently to pH fluctuations, and thus, to reduce the dissolution of hydroxyapatite, preventing the onset of caries lesion. Another factor is that this combination led to higher levels of F in the biofilm fluid, as well as the formation of $CaHPO_4^0$, HPO_4^{2-}, CaF^+ and HF^0 which may prevent demineralization and assist in dental remineralization. However, the experimental model presents some limitations, such as the limited number of microorganisms used, the absence of a dental substrate for biofilm formation on it, and the lack of a continuous salivary flow, as occurring under *in vivo* conditions. Such limitations, however, were necessary to minimize possible interferences that could be confounding factors in an initial study. In this sense, it would be interesting to develop new studies with other experimental models, such as biofilm microcosms, to confirm the present findings.

To sum up, it is possible to conclude that TMP significantly affects the biofilm, by increasing the concentration of F and P of the biofilm fluid, and by maintaining the pH of the medium close to the neutral values even after exposure to sucrose. Thus, the use of this phosphate combined with F, presents biofilm modifications that may interfere with the dynamics of dental caries.

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Table legend

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

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

Figures legends

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

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

Figure 3. Possible formations of $CaHPO_4^0$ (A), HPO_4^{-2} (B), CaF^+ (C), HF^0 (D) in the biofilm fluid before and after contact with the sucrose. Lower case letters present statistical difference between groups ($p < 0.05$).

Table

Table 1

	Groups								
	Control	**500 ppm F**	**1100 ppm F**	**0.25% TMP**	**0.5% TMP**	**1% TMP**	**0.25% TMP/F**	**0.5% TMP/F**	**1% TMP/F**
no-sucrose	$6.01^{a,A}$ (0.15)	$6.54^{b,A}$ (0.19)	$6.99^{c,A}$ (0.14)	$5.82^{a,A}$ (0.08)	$5.96^{a,A}$ (0.05)	$6.38^{b,A}$ (0.31)	$7.09^{c,A}$ (0.12)	$7.08^{c,A}$ (0.22)	$7.57^{d,A}$ (0.29)
Sucrose	$4.80^{a,B}$ (0.09)	$6.03^{b,d,B}$ (0.38)	$6.54^{c,B}$ (0.19)	$6.12^{b,B}$ (0.16)	$5.85^{d,A}$ (0.28)	$6.21^{b,A}$ (0.17)	$6.46^{c,B}$ (0.13)	$6.62^{c,B}$ (0.22)	$7.18^{e,B}$ (0.22)

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

Table 2.

Groups	*Degree of Saturation*			
	HA		CaF_2	
	no sucrose	sucrose	no sucrose	sucrose
Control	$0.80^{a,A}$ (0.12)	$-13.60^{a,B}$ (0.97)	$-3.43^{a,A}$ (0.15)	$-5.26^{a,B}$ (0.17)
500 ppm F	$2.84^{b,A}$ (0.32)	$-9.70^{b,B}$ (0.41)	$2.92^{b,A}$ (0.10)	$0.86^{b,B}$ (0.19)
1100 ppm F	$-25.16^{c,A}$ (0.36)	$-29.97^{c,B}$ (0.87)	$-2.44^{c,A}$ (0.02)	$-3.22^{c,B}$ (0.03)
0.25% TMP	$-0.87^{d,A}$ (0.22)	$-30.27^{c,B}$ (1.00)	$-3.64^{d,A}$ (0.15)	$-10.04^{d,B}$ (0.04)
0.5% TMP	$1.50^{a,A}$ (0.11)	$-30.14^{c,B}$ (1.36)	$-3.77^{d,A}$ (0.13)	$-10.11^{d,B}$ (0.18)
1% TMP	$4.06^{e,A}$ (0.26)	$-28.52^{d,B}$ (0.88)	$-3.75^{d,A}$ (0.11)	$-10.26^{d,B}$ (0.07)
0.25% TMP/F	$6.18^{f,A}$ (0.26)	$-5.84^{e,B}$ (1.09)	$3.15^{e,A}$ (0.08)	$0.72^{b,B}$ (0.34)
0.5% TMP/F	$6.75^{f,A}$ (0.25)	$-26.91^{f,B}$ (0.52)	$3.22^{e,A}$ (0.04)	$-3.94^{e,B}$ (0.05)
1% TMP/F	$8.70^{g,A}$ (0.19)	$-23.54^{g,B}$ (1.13)	$3.26^{e,A}$ (0.07)	$-3.76^{f,B}$ (0.05)

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

Figures

Figue 1

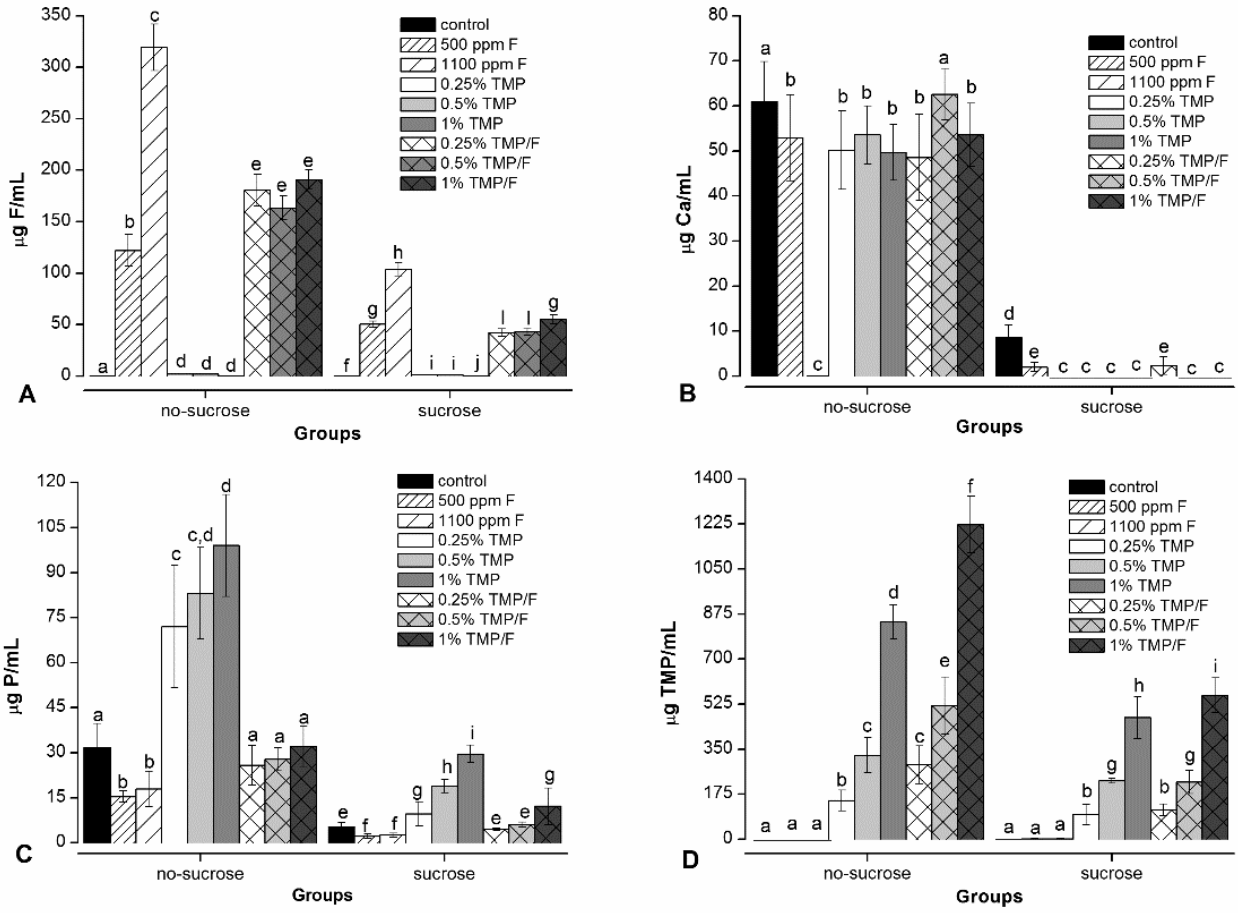


Figure 2.

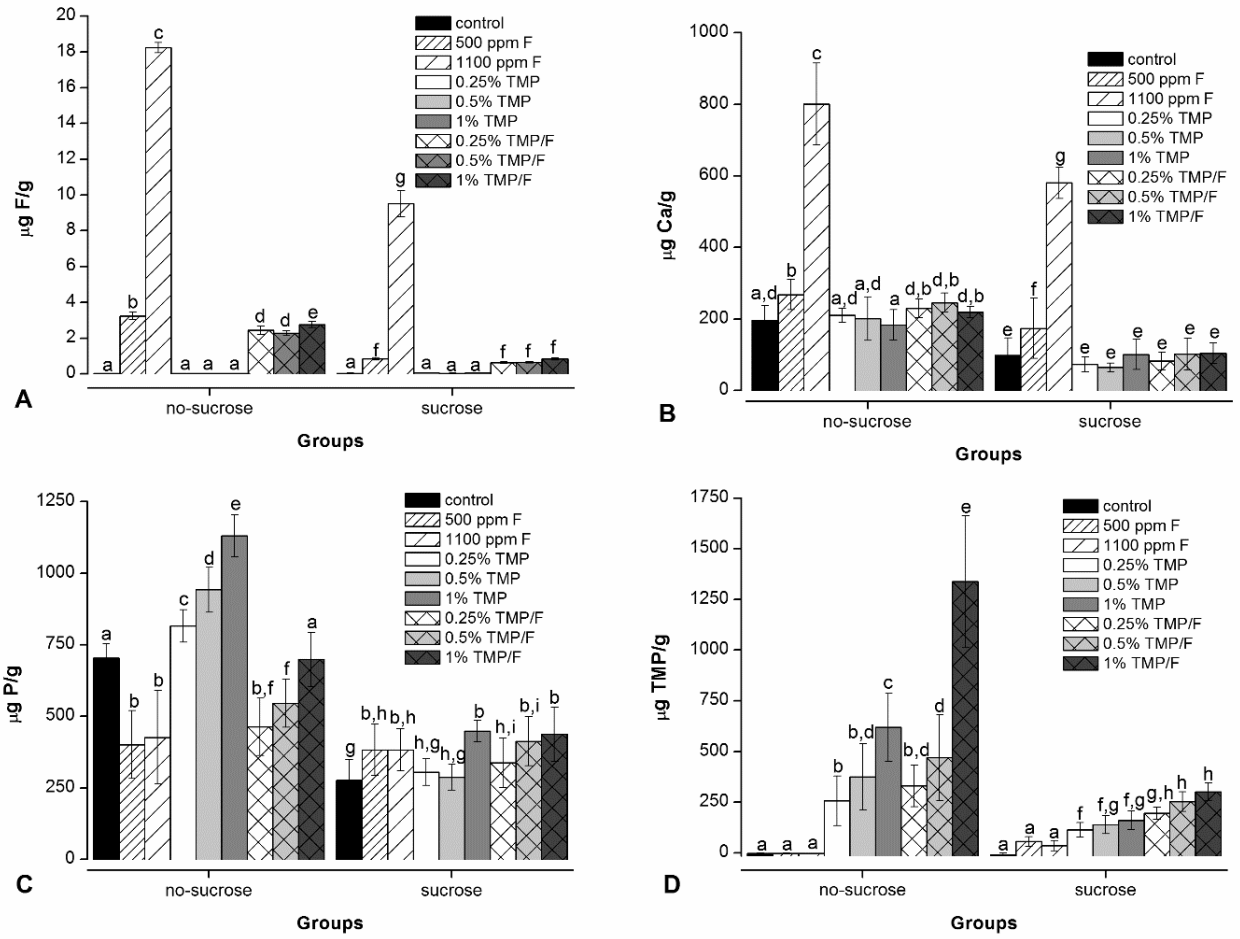
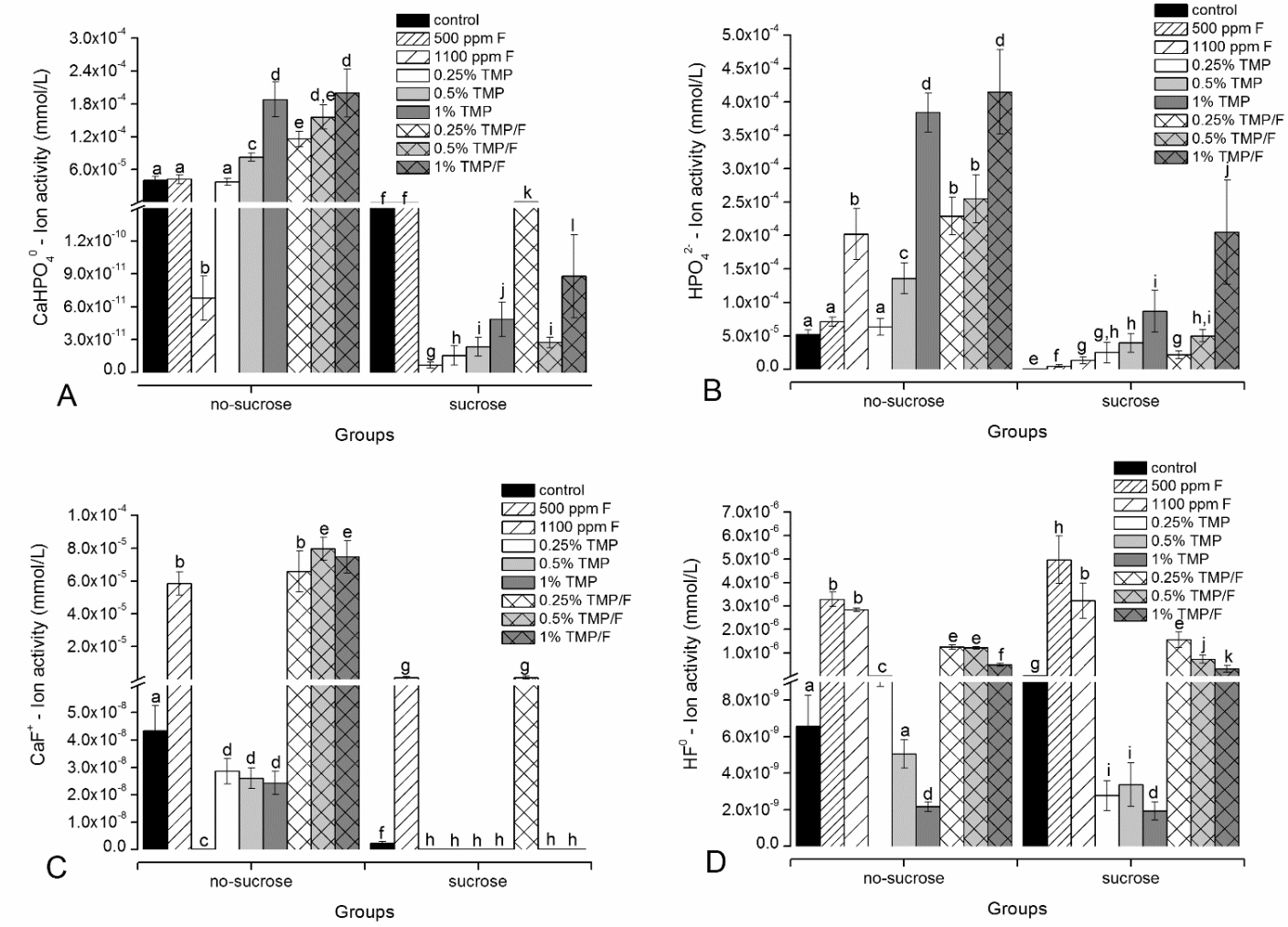

A
µg F/g
control
500 ppm F
1100 ppm F
0.25% TMP
0.5% TMP
1% TMP
0.25% TMP/F
0.5% TMP/F
1% TMP/F
no-sucrose
sucrose
Groups
B
µg Ca/g
C
µg P/g
D
µg TMP/g

Figure 3.


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

Anexos

ANEXO A

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ANEXO B

Future Microbiology

Future Medicine - Preparing your article

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Article sections

The following list provides notes on the key article sections; authors should consult the 'at-a-glance formatting checklist' to determine which sections are required for their submission.

Title

Concisely and clearly conveys the scope/novelty of the article; not more than 120 characters.

Author(s) names & affiliations

Including full name, address and e-mail.

Guidance on author sequence: Author sequence is at the authors' discretion; however, Future Medicine journals suggest following the recommendations in GPP3 Appendix Table 2 (http://www.ismpp.org/gpp3), whereby authors are listed either in order of the level of their contribution, or alphabetically. The corresponding author should always be indicated.

Guidance on a change of affiliation during writing: Where an author has changed their affiliation prior to the publication of an article, the affiliation should reflect where the major part of the work was completed. Current affiliation and contact information should be listed in an acknowledgement.

Abstract

Not more than 120 words; no references should be cited in the abstract. The abstract should highlight the importance of the field under discussion within the journal's scope, and clearly define the parameters of the article.

Structured abstract (for Research articles)

Not more than 120 words, broken down into Aims, Patients & Methods/Materials & Methods, Results and Conclusions. For authors presenting the results of clinical trials, the guidelines recommended by CONSORT should be followed when writing the abstract (http://www.consortstatement.org/), and the clinical trial registration number included at the end of the abstract, where available. Data deposition: where data have been deposited in a public repository, authors should state at the end of the abstract the data set name, repository name and number.

Keywords

Up to 10 keywords (including therapeutic area, mechanism[s] of action etc.) plus names of drugs and compounds mentioned in the text.

Body of the article

The article content should be arranged under relevant headings and subheadings to assist the reader.

Future perspective

The author is challenged to include speculative viewpoint on how the field will have evolved 5– 10 years from the point at which the article was written.

Executive summary

A series of bulleted summary points that illustrate the main topics or conclusions made under each of the main headings of the article.

Summary points (Research articles & Company profiles only)

8–10 bullet point sentences highlighting the key points of the article.

Financial disclosure/Acknowledgements

Disclosing any financial and/or material support that was received for the research or the creation of the work. Also disclosing any relationships any authors have (personal, academic or financial relationships that could influence their actions) or financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. If writing assistance has been used in the creation of the manuscript, this should also be stated and any sources of funding for such assistance clearly identified.

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For studies involving data relating to human or animal experimental investigations, authors should obtain appropriate institutional review board approval and state this within the article (for those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be followed). In addition, for investigations involving human subjects, authors should obtain informed consent from the participants involved and include an explanation of how this was obtained in the manuscript.

References

Key points

- Authors should focus on recent papers and papers older than 5 years should not be included except for an over-riding purpose.

- Primary literature references, and any patents or websites, should be numerically listed in the reference section in the order that they occur in the text (including any references that only appear in figures/tables/boxes).

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- Avoid citing a “personal communication” unless it provides essential information not available from a public source, in which case the name of the person and date of communication should be cited in the text, with written permission from the source.

- References should be denoted numerically and in sequence in the text, using Arabic numerals placed in square brackets, i.e., [12].

- Quote first six authors’ names. If there are more than six, then quote first three et al.

- Reference annotations: 6–8 references should be highlighted that are of particular significance to the subject under review as “* of interest” or “** of considerable interest”, along with a brief (1–2 line) synopsis.

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Format

- Author's names should appear without full stops in their initials
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- A full stop follows authors' names
- Journal name should be in italics and abbreviated to standard format
- Volume number followed by comma, not bold
- Page number range separated by a hyphen with no spaces, followed by the year in brackets, and then a full stop

Examples

Journal example: Fantl JA, Cardozo L, McClish DK et al. Estrogen therapy in the management of urinary incontinence in postmenopausal women: a meta-analysis. Obstet. Gynecol. 83(1), 12–18 (1994).

Book example: De Groat WC, Booth AM, Yoshimura N. Neurophysiology of micturition and its modification in animal models of human disease. In: The Autonomic Nervous System (Volume 6). Andrews WR (Ed.), Harwood Academic Publishers, London, UK, 227–289 (1993).

Meeting abstract example: Smith AB, Jones CD. Recent progress in the pharmacotherapy of diseases of the lower urinary tract. Presented at: 13th International Symposium on Medicinal Chemistry. Atlanta, GA, USA, 28 November–2 December 1994.

Patent example: Merck Frosst Canada, Inc. WO9714691 (1997). (Use the following formats for patent numbers issued by the World, US and European patent offices, respectively: WO1234567, US1234567, EP-123456-A).

Website example (organization homepage): US Food and Drug Association. www.fda.gov

Website example (specific webpage/document): American Cancer Society. Cancer Facts and Figures 2015 (2015). www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/index

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Reference annotations

Papers or of particular interest should be identified using one or two asterisk symbols:

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- ** = of considerable interest

Each of the chosen references should be annotated with a brief sentence explaining why the reference is considered to be of interest/particular interest.

Figures, tables, boxes & supplementary materials (incl. video)

Summary figures, tables and boxes are very useful, and we encourage their use in certain article types (see above section on Article types for details on which articles can include figures/tables/boxes). The author should include illustrations to condense and illustrate the information they wish to convey. Commentary that augments an article and could be viewed

as ‘stand-alone’ should be included in a separate box. An example would be a summary of a particular trial or trial series, a case study summary or a series of terms explained.

Figures, tables and boxes should be numbered consecutively according to the order in which they have been first cited in the text.

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- File format: All figures, tables and boxes should be submitted in an editable format. For figures that will be included without editing (i.e., photos, imaging data, etc.) please submit as a .jpeg, .pdf or .tiff. Other figures (i.e., graph/bar charts or complex illustrations) should ideally be provided as Adobe Illustrator files (.ai or .eps) if possible, otherwise as a .jpeg, .pdf or .tiff. Tables/boxes should be provided as Microsoft Word, Microsoft Excel or Adobe Illustrator files, and must be editable. If you are uncertain whether the format of your files is appropriate, please check with the Journal Editor.

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- Draw small peptides (up to five amino acids) in full; use amino acid abbreviations (Gly, Val, Leu, etc.) for larger peptides.

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Blood pressures should be in millimeters of mercury.

Any other units should be reported using the International System of Units (SI) where possible.

Statistics

Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to judge its appropriateness for the study and to verify the reported results.

When possible, appropriate indicators of measurement error or uncertainty (such as confidence intervals) should be included.

Please define any statistical terms, abbreviations and symbols used.

ANEXO C

Caries Research

Guidelines for Authors
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Aims and Scope

'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.
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Manuscripts written in English should be submitted online.

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(Editor-in-Chef, Caries Research)
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The Henry Wellcome Laboratories for Microbiology and Salivary Research
KCL Dental Institute, Floor 17, Guys Tower
London Bridge SE1 9RT (UK)
Tel. +44 2071887465
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Copies of any 'in press' papers cited in the manuscript must accompany the submission. Manuscripts reporting on clinical trials must be accompanied by the CONSORT checklist (see below).

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Whether intentional or not, plagiarism is a serious violation. We define plagiarism as a case in which a paper reproduces another work with at least 25% similarity and without citation. If evidence of plagiarism is found before/after acceptance or after publication of the paper, the author will be offered a chance for rebuttal. If the arguments are not found to be satisfactory, the manuscript will be retracted and the author sanctioned from publishing papers for a period to be determined by the responsible Editor(s).

Conditions

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Types of Papers

Original papers or Short Communications are reports of original work (including systematic reviews and meta-analyses). Both have the structure outlined below but for Short Communications the abstract should be less than 100 words and the manuscript should not exceed 3 printed pages, equivalent to about 9 manuscript pages (including tables, illustrations and references).

Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope. Reviews are not subject to page charges.

Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.

Letters to the Editor, commenting on recent papers in the journal, are published occasionally, together with a response from the authors of the paper concerned.

Preparation of Manuscripts

Text should be one-and-a-half-spaced, with wide margins. All pages and all lines must be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes.

Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Lines in the manuscript must be numbered consecutively from the title page until the last page. Submissions which do not conform to these simple guidelines will be returned to the author.

Title page: The first page of each manuscript should show, in order:
 the title, which should be informative but concise;
 the authors' names and initials, without degrees or professional status, followed by their institutes;
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(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

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