IGOR RENAN ZEN

Efeito de soluções ou dentifrícios contendo trimetafosfato de sódio, xilitol, eritritol e fluoreto, em diferentes associações, sobre biofilmes de interesse cariogênico

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RESUMO GERAL

Este estudo avaliou o efeito de dentifrícios ou soluções contendo trimetafosfato de sódio(TMP), xilitol(X), eritritol(E) e fluoreto(F), em diferentes associações, sobre cepas e biofilmes cariogênicos. Três subprojetos (SP1, SP2 e SP3) apresentaram os objetivos: SP1) Avaliar o efeito de dentifrícios contendo "TMP(0,25%)", "X(16%)", "E(4%)", "F(200 e 1100 ppm)" sozinhos ou em diferentes associações, sobre cepas isoladas de *Streptococcus mutans*(SM), *Lactobacillus casei*(LC), *Actinomyces israelii*(AI) e *Candida albicans*(CA). SP2) Avaliar o efeito de soluções contendo "TMP"(0,075%), "X"(4,8%), "E"(1,2%), "F"(60 e 330 ppm) e saliva artificial pura, sozinhos ou em diferentes associações sobre biofilmes mistos de SM e CA. SP3) Avaliar o efeito das mesmas soluções de SP2 sobre biofilmes microcosmos patogênicos com a incorporação ou não de SM. No SP1, cepas de SM, LC, AI e CA foram incorporadas ao meio de BHI-ágar, vertidas em placas, realizados poços no ágar e diferentes diluições de slurries dos dentifrícios foram adicionados. Os halos foram medidos com paquímetro digital. A análise estatística se deu por ANOVA dois critérios, e teste de Tukey HSD ($p<0,05$). Para SM, o maior halo foi observado por "200F+TMP" em todas as diluições, seguido por "200F+X+E". Para LC, a tendência mostrou inibição microbiana promovida pelos polióis, potencializado pela associação com os outros compostos. Para AI, observou-se uma tendência menos definida. Para CA, o dentifrício experimental "200F+X+E+TMP" foi mais eficaz que os outros. No SP2, as mesmas soluções e grupos do SP1 foram usados a uma concentração final de 30% do valor inicial dos dentifrícios. Biofilmes mistos de SM e CA foram cultivados na presença contínua desses ativos e avaliou-se a quantificação de células viáveis (UFCs), biomassa total, atividade metabólica e componentes da matriz extracelular. A análise estatística se deu por ANOVA um

critério e teste de Tukey HSD ($p<0,05$). As contagens de UFCs foram afetadas pelo F, enquanto a biomassa e atividade metabólica pelo TMP. Adicionalmente, observou-se efeito sinérgico desses ativos. Os polióis tiveram efeitos mais pronunciados nos carboidratos da matriz extracelular, com pouca ou nenhuma ação nas demais variáveis. A associação dos quatro ativos promoveu aumento no efeito antibiofilme, e foi afetado por F e/ou TMP, com pouco efeito dos polióis isoladamente. No SP3, biofilmes microcosmos foram formados em um modelo de biofilme de alto rendimento com ou sem a incorporação da cepa de SM. As mesmas soluções e concentrações de SP2 estavam constantemente presentes no meio de cultura. Analisou-se as UFCs e produção de acido lático dos biofilmes. Os dados foram analisados por ANOVA ou Kruskal-Wallis, e Student-Newman-Keuls ($p<0,05$). O grupo “60F+TMP” produziu quantidades de ácido lático significativamente menor, e apresentou reduções na contagem total de UFCs em biofilmes microcosmos, incorporados ou não com SM, comparado ao grupo controle. O grupo experimental promoveu diminuições sobre os parâmetros analisados. A associação de “F+TMP” e o grupo experimental reduziram as contagens de UFCs total e de SM, e a produção de ácido lático por biofilmes microcosmos derivados de saliva. Os resultados permitiram concluir que a associação dos quatro compostos ativos e “F+TMP” apresentaram reduções em todos os parâmetros avaliados.

Palavras-chaves: Fluoretos. Biofilmes. Fosfatos. Xilitol. Eritritol.

Zen, IR. **Effect of solutions or dentifrices containing sodium trimetaphosphate, xylitol, erythritol and fluoride, in different associations, on biofilms of cariogenic interest**.128 f. [thesis]. Araçatuba: UNESP – São Paulo State University;2022.

GENERAL ABSTRACT

This study evaluated the effect of dentifrices or solutions containing sodium trimetaphosphate(TMP), xylitol(X), erythritol(E) and fluoride(F), in different associations, on cariogenic strains and biofilms. Three subprojects (SP1, SP2 and SP3) presented the objectives: SP1) To evaluate the effect of dentifrices containing “TMP(0.25%)”, “X(16%)”, “E(4%)”, “F(200 and 1100 ppm)” alone or in different associations, on isolated strains of *Streptococcus mutans*(SM), *Lactobacillus casei*(LC), *Actinomyces israelii*(AI) and *Candida albicans*(CA). SP2) Evaluate the effect of solutions containing “TMP”(0.075%), “X”(4.8%), “E”(1.2%), “F”(60 and 330 ppm) and pure artificial saliva, alone or in different associations on mixed SM and CA biofilms. SP3) To evaluate the effect of the same SP2 solutions on pathogenic microcosm biofilms with or without the incorporation of SM. In SP1, SM, LC, AI and CA strains were incorporated into the BHI-agar medium, poured into plates, wells were made in the agar, and different dilutions of dentifrice slurries were added. The halos were measured with a digital caliper. Statistical analysis was performed by two-way ANOVA and Tukey HSD test ($p<0.05$). For SM, the largest halo was observed by “200F+TMP” in all dilutions, followed by “200F+X+E”. For LC, the trend showed microbial inhibition promoted by polyols, potentiated by the association with the other compounds. For AI, a less defined trend was observed. For CA, the experimental dentifrice “200F+X+E+TMP” was more effective than the others. In SP2, the same solutions and groups of SP1 were used at a final concentration of 30% of the initial value of the dentifrices. Mixed biofilms of SM and CA were cultured in the continuous presence of these actives, and the quantification of viable cells (CFUs), total biomass, metabolic activity and extracellular matrix components were evaluated. Statistical analysis was performed by one-way ANOVA and Tukey HSD test ($p<0.05$). CFU counts were affected by F, while biomass and

metabolic activity by TMP. Additionally, a synergistic effect of these actives was observed. Polyols had more pronounced effects on extracellular matrix carbohydrates, with little or no action on other variables. The association of the four actives promoted an increase in the antibiofilm effect and was affected by F and/or TMP, with little effect of polyols alone. In SP3, microcosm biofilms were formed in a high-throughput biofilm model with or without the incorporation of the SM strain. The same SP2 solutions and concentrations were constantly present in the culture medium. The CFUs and lactic acid production of biofilms were analyzed. Data were analyzed by ANOVA or Kruskal-Wallis and Student-Newman-Keuls ($p<0.05$). The “60F+TMP” group produced significantly lower amounts of lactic acid and showed reductions in total CFU counts in microcosm biofilms, whether or not incorporated with SM, compared to the control group. The experimental group promoted decreases in the analyzed parameters. The association of “F+TMP” and the experimental group reduced total and SM CFU counts and lactic acid production by saliva-derived microcosm biofilms. The results allowed us to conclude that the association of the four active compounds and “F+TMP” showed reductions in all evaluated parameters.

Key words: Fluorides. Biofilms. Phosphates. Xylitol. Erythritol.

LISTA DE SIGLAS

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1100F	1100 ppm F
200F	200 ppm F
200F+TMP	200 ppm F + trimetafosfato de sódio/sodium trimetaphoshate
200F+X+E	200 ppm F + xilitol + eritritol/ 200 ppm F + xylitol + erythritol
AI/*A. israelii*	*Actinomyces israelii*
ANOVA	Analise de Variância/Analysis of Variance
AS	Saliva artificial/Artificial saliva
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BPW	Água Peptonada Tamponada/ Buffered Peptone Water
CA/*C.albicans*	*Candida albicans*
Ca^{+}	Cálcio/Calcium
CaF_2	Fluoreto de Cálcio/Calcium Fluoride
Cm	Centímetros/Centimeters
CO_2	Dióxido de Carbono/Carbon dioxide
CV	Cristal Violeta/Crystal Violet
SDA	Sabouraud Dextrose Agar
E	Eritritol/Erythritol
e.g./exempli gratia	Por exemplo/ For example
EXP/200F+TMP+X+E	Grupo experimental/Experimental Group / 200 ppm F + Trimetafosfato de sódio + Xilitol + Eritritol/ 200 ppm F + Sodium trimetaphosphate + Xylitol + Erythritol
F	Flúor/Fluoride
g	Gramas/Grams
Gtfs	Glicosiltransferases/Glycosyltransferase

h	Hora/Hour
H_2	Hidrogênio/Hydrogen
HSD	Diferença Significativa Honesta/Honestly Significant Difference
i.e./id est	Isto é/That is
KCl	Cloreto de Potássio/ Potassium Cloride
L/l	Litros/Liters
LC/*L.casei*	*Lactobacillus casei*
Log_{10}	Logaritmo na base 10/ Logarithm, base 10
Mg	Miligramas/Miligrams
Mg^+	Magnésio/Magnesium
Min	Minutos/Minutes
mL	Mililitros/Mililiters
mm	Milímetros/Milimetres
Mmol	Milimol
Modelo AAA	Amsterdam Active Attachment Model
n	Número/Number
N_2	Nitrogênio/Nitrogen
NaCl	Cloreto de Sódio/Sodium Cloride
NaF	Fluoreto de Sódio/Sodium Fluoride
NaOH	Hidróxido de Sódio/Sodium Hidroxide
NC	Controle Negativo/Negative Control
Nm	Nanômetro/Nanometer
ºC	Graus Celsius/ Degrees Celcius
p	Probabilidade/Probability
pH	Potencial de Hidrogênio/Potential of Hydrogen
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
ppm	Parte por milhão/Parts per million
Rpm	Rotação por minuto/Revolutions per minute
SAB	Ágar Sacarose Bacitracina/ Sucrose Agar Bacitracin
SD	Desvio Padrão/Standard Deviation

SDB	Sabouraud Dextrose Broth
SM/*S. mutans*	*Streptococcus mutans*
TMP	Trimetafosfato de sódio/Sodium trimetaphosphate
TMP+X+E	Trimetafosfato de sódio + xilitol + eritritol/ Sodium trimetaphosphate + xylitol + erythritol
TSBA	Ágar Sangue /Tryptic Soy Agar Blood
UFC/CFU	Unidades de Formação de Colônias/Colony-Forming Unit
v/v	Volume por volume/Volume per volume
VU	Vrije Universiteit
W	Watts
X	Xilitol/Xylitol
X+E	Xilitol + eritritol/ Xylitol + erythritol
XTT	2,3-bis (2-methoxy-6 4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide
µL	Microlitros/microliters

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INTRODUÇÃO GERAL

INTRODUÇÃO GERAL

A cárie dentária é uma doença que afeta mais de 3,5 bilhões de pessoas durante a dentição permanente e mais de 620 milhões de crianças durante a dentição primária (Kassebaum et al., 2017; Phantumvanit et al., 2018). Esta doença é mediada por biofilmes formados por um consórcio de diferentes micróbios. Também é modulada pela dieta na presença de açúcares fermentáveis, não transmissível e é considerada multifatorial (Machiulskiene et al., 2020). Um dos principais agentes etiológicos da cárie dentária é a bactéria gram-positiva *Streptococcus mutans* (*S. mutans*), devido a 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 (Falsetta et al., 2014; Lemos et al., 2019), levando à formação do biofilme dentário, o qual é definido como uma comunidade microbiana organizada, aderida a superfícies vivas ou inertes e envolvida por uma matriz extracelular produzida pelas células (Bowen, Burne, Wu, & Koo, 2018).

No biofilme, os microrganismos apresentam-se embebidos em uma matriz extracelular composta por glicoproteínas e polissacarídeos (Lemos et al., 2019). Inicialmente, várias adesinas das bactérias odontopatogênicas interagem com as glicoproteínas salivares da película adquirida na superfície dos dentes através de cátions bivalentes. Na presença de sacarose, as bactérias aderem-se firmemente à superfície do dente como resultado da produção de exopolissacarídeos (glucanos) por meio da atividade da glicosiltransferase (*Gtfs*). Sendo assim, o acúmulo de biofilme faz com que o *S. mutans* metabolize eficientemente a sacarose (açúcar ou polímeros) para produzir grande quantidade de ácido láctico, capaz de solubilizar o componente mineral do dente e iniciar o processo de cárie (Lemos et al., 2019). Além do *S. mutans*, a cavidade bucal é habitada por mais de 700 diferentes espécies de microrganismos, dentre esses, espécies bacterianas como de *Lactobacillus*, *Actinomyces* e fúngicas como *Candida* sp.. *Candida albicans* (*C. albicans*) é o fungo mais comumente encontrado na cavidade oral, que pode ser um fator de risco para o desenvolvimento de cárie dentária (Kilian et al., 2016). A presença de *C. albicans* é importante na cárie na infância, uma vez que contribui para a patogênese em crianças cárie-ativas (Menon, Scoffield, Jackson, & Zhang, 2022).

O uso dos dentifrícios contendo fluoreto (F) atrelado a escovação dos dentes é considerado o melhor método preventivo da cárie dentária, visto que associa a remoção ou desorganização periódica do biofilme dental com as propriedades cariostáticas do F (Walsh, Worthington, Glenny, Marinho, & Jeroncic, 2019). Sendo assim, o íon flúor tem seu efeito por meio da manutenção da concentração de flúor na saliva devido ao uso frequente dos dentifrícios, e pela formação de produtos da reação esmalte-dentina com fluoreto, formando o mineral fluoreto de cálcio (CaF_2), que, depositado no biofilme em lesões iniciais de cárie, é capaz de evitar a progressão da mesma (Buzalaf, Pessan, Marques Honório, & Ten Cate, 2011).

A suplementação com sais de fosfato tem sido uma possibilidade para aumentar a efetividade dos dentifrícios fluoretados. Estudos *in vitro* e *in situ* demonstraram que dentifrícios com concentração reduzida de F suplementados com trimetafosfato de sódio (TMP) apresentam efetividade semelhante a de um dentifrício convencional (1.100 µg F/g) (Takeshita, Danelon, Castro, Cunha, & Delbem, 2016; Takeshita, Danelon, Castro, Sassaki, & Delbem, 2015). Além disso, a concentração de F e cálcio no biofilme formado na presença destes dentifrícios foi semelhante à observada para um dentifrício convencional (Takeshita et al., 2015). No entanto, o mecanismo pelo qual soluções contendo TMP, associados ou não ao F, atuam no biofilme ainda é incerto.

Além desses sais de fosfato, existem produtos naturais que são fontes de agentes terapêuticos, e que podem ser utilizados na prevenção da cárie dentária, pois apresentam efeito sobre os microrganismos do biofilme. O xilitol e o eritritol são polióis utilizados como substitutos do açúcar em muitos produtos de cuidados bucais, pois não são cariogênicos e apresentam efeitos favoráveis sobre a saúde bucal (Kauko Mäkinen, 2000; Runnel et al., 2013). A literatura mostra que o xilitol diminui a acidogenicidade e volume do biofilme, e é capaz de inibir o crescimento de *S. mutans* (Kauko Mäkinen, 2000). Da mesma forma, o eritritol parece apresentar mecanismos semelhantes ao do xilitol e promove a redução dos microrganismos patogênicos relacionados à cárie dentária (*S. mutans*) (Park et al., 2014).

Diante de todo contexto supracitado, e tendo em vista a eficácia dos ativos sozinhos, os objetivos do presente estudo foram: 1) Avaliar o efeito de dentifrícios contendo trimetafosfato de sódio (TMP), xilitol (X), eritritol (E) e fluoreto (F), em diferentes associações, sobre cepas isoladas de *Streptococcus mutans*, *Lactobacillus*

casei, *Actinomyces israelii* e *Candida albicans* na formação de halos de inibição. 2) Avaliar o efeito de soluções contendo trimetafosfato de sódio (TMP), xilitol (X), eritritol (E) e fluoreto (F), em diferentes associações sobre biofilmes mistos de *Streptococcus mutans* e *Candida albicans*. 3) Avaliar o efeito de soluções contendo trimetafosfato de sódio (TMP), xilitol (X), eritritol (E) e fluoreto (F), em diferentes associações sobre biofilmes microcosmos (polimicrobianos) suplementados ou não com *Streptococcus mutans*.

Os dentifrícios foram testados no capítulo 1 e as soluções nos capítulos 2 e 3, sendo respectivamente:

Dentifrícios: Trimetafosfato de sódio (0,25%), F (200 ppm), xilitol (16%) e eritritol (4%), 1100 ppm F (controle positivo) e placebo (sem adição de nenhum composto ativo).

Soluções: Trimetafosfato de sódio (0,075%), F (60 ppm), xilitol (4,8%) e eritritol (1,2%), 330 ppm F (controle positivo) e Saliva artificial ou McBain puro (controle negativo).

Para alcançar os objetivos propostos, o presente estudo foi dividido em três capítulos, como detalhado abaixo:

- Capítulo 1: “Antimicrobial effect of low-fluoride toothpastes containing polyphosphate and polyols” (artigo submetido ao periódico Saudi Dental Journal);
- Capítulo 2: “Evaluation of solutions containing fluoride, sodium trimetaphosphate, xylitol and erythritol on dual-species biofilms” (artigo submetido ao periódico Biofouling);
- Capítulo 3: “Assessment of the effects of fluoride, sodium trimetaphosphate, xylitol and/or erythritol on saliva-derived microcosm biofilms” (artigo formatado nas normas do periódico Archives of Oral Biology).

As referências da Introdução Geral encontram-se no anexo A.

CAPÍTULO 1

Antimicrobial effect of low-fluoride toothpastes containing polyphosphate and polyols

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Este artigo segue as normas do periódico Saudi Dental Journal (Anexo B)

Antimicrobial effect of low-fluoride toothpastes containing polyphosphate and polyols

Abstract

This study evaluated the antimicrobial effect of toothpastes containing 200 ppm fluoride (200F), xylitol (X, 16%), erythritol (E, 4%), and sodium trimetaphosphate (TMP, 0.25%), alone or in different associations, against *Streptococcus mutans* (SM), *Lactobacillus casei* (LC), *Actinomyces israelii* (AI) and *Candida albicans* (CA). Suspensions of the microorganisms were added to BHI Agar medium. Five wells were made on each plate to receive toothpaste suspensions at different dilutions. Toothpastes containing no actives (Placebo) or 1100 ppm F (1100F) were used as negative and positive controls. 2-way ANOVA and Tukey's HDS test were used ($p<0.05$). For SM, the largest halo was for 200F+TMP at all dilutions, followed by the 200F+X+E toothpaste. For LC, the overall trend showed that the polyols effectively inhibited microbial growth, and the association with the other compounds enhanced such effects. For AI, a less defined trend was observed. For CA, the experimental toothpaste (200F+X+E+TMP) was consistently more effective than the other treatments and followed by 200F+X+E. The association of polyols and TMP in a low-fluoride toothpaste effectively reduced the growth of cariogenic microorganisms (SM, CA, and LC), suggesting that this formulation could be an interesting alternative for children due to its low fluoride content.

KEYWORDS: Toothpastes; Sugar Alcohols; Fluorides; Polyphosphates
Total words: 2.497

Declarations of interest: none

Introduction

Dental caries is a chronic, biofilm-modulated disease, which has increasingly affected children worldwide (Kassebaum et al., 2017), being associated with an increase in fermentable carbohydrates intake, and deficient removal or disorganization of dental biofilm. It is a sucrose-dependent biofilm disease, whose onset is related to the interaction of acidogenic and aciduric microorganisms in the oral cavity (Machiulskiene et al., 2020).

Among the main microorganisms involved, *Streptococcus mutans* stand out for their ability to colonize dental surfaces, metabolize carbohydrates, and produce lactic acid (Klein et al., 2015). Other bacteria are related to the disease's onset and progression, including *Lactobacillus* and *Actinomyces* (Hamada & Slade, 1980). In addition, the fungus *Candida albicans* has been reported to contribute to the formation and development of cariogenic biofilms, especially in dentine cavities, as its proteolytic enzymes are able to break down collagen molecules (Falsetta et al., 2014; Menon et al., 2022).

The association of mechanical and chemical methods is an effective method to reduce biofilm formation (Sampaio et al., 2020; Valkenburg, Else Slot, & Van der Weijden, 2020; Walsh et al., 2019). Among them, toothpastes present in their composition active agents that have mineralizing and/or antimicrobial action, such as Fluoride (F) (Valkenburg et al., 2020; Walsh et al., 2019). Furthermore, the addition of phosphate salts to F-containing toothpastes has been shown to boost the effects of F. *In vitro* and *in situ* studies have shown that low-F toothpastes containing sodium trimetaphosphate (TMP) have similar effects to those of a conventional formulation (1100 ppm F) on enamel de- and re-mineralization (Takeshita et al., 2016, 2015), and on F and calcium levels in the dental biofilm (Takeshita et al., 2015).

In addition to the strategies above, products of natural origin, such as polyols, are used as sugar substitutes, which have ability to reduce the growth and attachment of *S. mutans* to surfaces and reduce the volume of dental biofilm (E. Söderling & Pienihäkkinen, 2022). Literature shows a synergism between xylitol and F on the inhibition of acid production by *S. mutans*, with significantly higher effects than the actives alone (Maehara, Iwami, Mayanagi, & Takahashi, 2005; Kauko K. Mäkinen, 2011). Furthermore, erythritol has a similar effect to xylitol and its action

seems to cause direct changes in the metabolism of caries-related microorganisms (De Cock et al., 2016; Janus et al., 2017).

Considering the advantages of the above-mentioned strategies used alone, and the need to develop safer and more effective formulations for children, this study assessed *in vitro* the antimicrobial effect of toothpastes containing F at low concentration (200 ppm), xylitol, erythritol, and TMP, alone or in different associations on the inhibition of *Streptococcus mutans* (*S. mutans*), *Lactobacillus casei* (*L. casei*), *Actinomyces israelii* (*A. israelii*) and *Candida albicans* (*C. albicans*) growth. The study's null hypothesis was that the antimicrobial effects of the toothpastes associating two or more compounds would not be significantly different from the toothpastes containing the isolated actives.

Material and methods

The toothpastes were produced by Xlear Incorporation, American Fork, UT, USA, and contained in their composition the following actives: xylitol (X: 16%), erythritol (E: 4%), sodium trimetaphosphate (TMP: 0.25%), and sodium fluoride at 200 ppm F, alone or in different combinations. Formulations without any actives (placebo) or containing 1100 ppm F were also manufactured as controls.

a) Placebo (without any active compound; negative control toothpaste);
b) 1100 ppm F (positive control toothpaste);
c) 200 ppm F (200F);
d) TMP (0.25%);
e) 200F+TMP;
f) Xylitol (16%);
g) Erythritol (4%);
h) Xylitol+erythritol;
i) 200F+xylitol+erythritol;
j) TMP+xylitol+erythritol;
k) 200F+TMP+xylitol+erythritol (experimental toothpaste).

2.1.1 Preparation of toothpaste slurries

Toothpaste slurries were produced by dispersing 10 g of each toothpaste in 10 mL of sterile deionized water (1:1). Serial dilutions of the slurry were made using sterile deionized water, to obtain four additional dilutions at 1:2, 1:4, 1:8, and 1:16 (Malhotra & Shashikiran, 2017).

2.1.2 Antimicrobial assays

Agar diffusion assays were performed with the reference strains of the American Type Culture Collection (ATCC): *C. albicans* (ATCC 10231), *S. mutans* (ATCC 25175) *A. israelii* (ATCC 10048) and *L. casei* (ATCC 393) (Malhotra & Shashikiran, 2017). These were reactivated from their original cultures in Sabouraud Dextrose Agar (SDA; Difco, Le Pont de Claix, France) for *C. albicans*, and in Brain and Heart Infusion Agar (BHI Agar; Difco) for *S. mutans, A. israelli*, and *L. casei*, and incubated in 5% CO_2 at 37 ºC, during 24 hours. Following, five colonies of each species were individually added to BHI broth and incubated at 37 ºC for 18-24 hours. Aliquots of 300 µL of each bacterial suspension (optical density of 0.6, and absorbance of 550 nm - 10^7 CFU/ml for *C. albicans* and 10^8 CFU/ml for *S. mutans*, *A. israelii* and *L. casei*) was homogenized with 15 mL of BHI-agar at 45 ºC. Subsequently, equidistant wells (n = 5) were made on agar using sterilized cylinders (4 mm in diameter) and filled with 80 µL of the slurries of each toothpaste, at dilutions of 1:1, 1:2, 1:4, 1:8 and 1:16 (Malhotra & Shashikiran, 2017). The plates were kept for 2 hours at room temperature to allow the solutions to diffuse, and then incubated at 37 ºC for 24 hours. The experiments were performed in triplicate. For each inhibition halo, three independent measures were performed with the aid of a digital caliper (accuracy 0.01 mm) (MitutoyoCD-15B) (Malhotra & Shashikiran, 2017).

2.1.3 Statistical analysis

Data were analyzed using the STATISTICA software (version 8.0). The data passed normality and homogeneity tests and were submitted to two-way Analysis of Variance (ANOVA), considering as variation factors toothpastes and dilutions, followed by the Tukey's HSD test, adopting a significance level of 5%.

Results

Overall, for *S. mutans* the largest inhibition halo was observed for 200F+TMP in all dilutions. Nonetheless, the effects of the polyols used together, as well as their association with 200 ppm F promoted larger inhibition halos compared with the other groups, with sustained effects for all the dilutions (Table 1).

For *L. casei*, the general pattern was that the polyols, alone or coadministered, were effective in inhibiting microbial growth, and their association with the other actives potentiated the antimicrobial effects (Table 2). In this sense, larger inhibition halos were observed for toothpastes containing both polyols and F (at most all the dilutions), both polyols and TMP (for the dilutions 1:2, 1:4 and 1:8), and for the experimental toothpaste (at 1:1, 1:2 and 1:4 dilution).

For *A. israelii*, no defined trend was observed for treatments at 1:1, 1:2, and 1:4 dilutions (Table 3). For the remaining dilutions (1:8 and 1:16), toothpaste containing 200 ppm F promoted the largest halos compared with the other treatments.

Conversely for *C. albicans*, the experimental toothpaste (containing all actives) promoted a consistently higher inhibitory effect than the other treatments for all dilutions (except for 1:8) and followed by 200F+X+E (Table 4).

Discussion

In this study, the antimicrobial effect of toothpastes containing 200 ppm F, xylitol (16%), erythritol (4%), and TMP (0.25%), isolated or in different associations on *S. mutans, A. israelii, L. casei* and *C. albicans*, was determined using an *in vitro* screening model. The results show that the toothpastes containing two or more actives promoted significantly larger inhibition halos than the isolated compounds for most of the pairwise comparisons, whose effects depended on the strain analyzed. Thus, the null hypothesis was partially rejected.

S. mutans was one of the strains chosen due to its direct involvement in dental caries onset and progression. For this strain, while toothpastes containing TMP alone or F at low concentrations promoted only modest antimicrobial effects, the largest inhibition halos were achieved by the association between 200 ppm F+TMP, for all dilutions (Table 1). These findings show the synergism between the two compounds, which is in agreement with CFU data of *C. albicans* and *S. mutans* (Cavazana et al., 2019). Furthermore, this association was shown to reduce the total biofilm biomass, extracellular matrix components, and also promoted the highest pH values both before and after cariogenic challenges (Cavazana et al., 2019, 2020). *In vitro* and *in situ* studies have also shown that the association between F and TMP leads to synergistic effects on de- and re-mineralization (Manarelli, Delbem, Lima, Castilho, & Pessan, 2014; Takeshita et al., 2016, 2015), reason by which such combination was assessed in the present study.

On the other hand, xylitol and/or erythritol promoted modest reductions in *S. mutans* growth. Literature reports that xylitol affects *S. mutans* cells by inhibiting glycolytic enzymes, which leads to reduction of growth and acid production (Söderling & Hietala-Lenkkeri, 2010). For erythritol, it appears to suppress the maturation of oral biofilms and prevent the development of dysbiosis (Janus et al., 2017). Despite the reasons for the small effects of the polyols on *S. mutans* in the present study are not apparent, it is possible that the effect of these actives on this bacterium would be related to their antibiofilm action (*e.g.*, extracellular polysaccharides production) rather an inhibitory effect.

As for *Lactobacillus casei*, conflicting evidence on the effects of xylitol is available from clinical studies. While some authors showed that xylitol's continued use promoted reductions in *Lactobacillus* counts in saliva (Mäkinen et al., 1995;

Mäkinen et al., 2008; Mäkinen et al., 1996), others showed that the use of xylitol-containing gum (Söderling et al., 2011) or toothpaste (Maden, Altun, Ozmen, & Basak, 2018) did not reduce *Lactobacillus* counts compared with control groups. The different methodologies, vehicles of administration, and study duration hinder a direct comparison between studies, as these variables may have impacted the results. In the present study, the largest inhibition zone was observed for the experimental toothpaste (at 1:1 dilution), followed by 1100F and 200F+X+E. Such effects might have resulted from xylitol's action on this strain following a similar mechanism to that described for *S. mutans*, in which is related to the phosphotransferase system that inhibits the conservation of the bacteria and further growth (Hausman, Thompson, & London, 1984; Helanto, Aarnikunnas, Palva, Leisola, & Nyyssölä, 2006; London & Hausman, 1982; Radmerikhi, Azul, Fajardo, & Formantes, 2013).

Regarding *Actinomyces israelii*, no striking pattern was observed among the groups on the inhibition halos compared with the placebo toothpaste, for any of the tested dilutions. This is in accordance with previous results showing that xylitol in the presence of glucose did not inhibit the growth of *Actinomyces* (Vadeboncoeur, Trahan, Mouton, & Mayrand, 1983). This fact can be explained by the low acidogenicity and acid tolerance of this strain (Tanzer, Livingston, & Thompson, 2001). They also reflect the status of non-cariogenic microorganisms compared with *Streptococcus mutans* and *Lactobacillus casei* (Tanzer et al., 2001; Yadav & Prakash, 2017).

In contrast, *Candida albicans* has recently been used in *in vitro* biofilm protocols to reproduce conditions that better resemble those of cariogenic biofilms *in vivo* (Cavazana et al., 2019, 2020) as this fungus has been identified in biofilms collected from individuals presenting cavitaded caries lesions (Menon et al., 2022). In the present study, the highest inhibition halos were promoted by the experimental toothpaste (at 1:1, 1:2, and 1:4 dilutions; table 4), so that the simultaneous action of all compounds could explain such effects. Previous studies have shown that xylitol presents an inhibitory effect on *C. albicans* growth (Talattof, Azad, Zahed, & Shahradnia, 2018), which is due to an impaired nutrient absorption required to maintain yeast viability. The inability of *C. albicans* cells in catabolizing or excreting xylitol products accumulated in the cytoplasm also explains such effects, as such products lead to an increased osmotic strength and cell swelling (Ameglio, Di Giorgio, Terzaroli, & Gandolfo, 1990; Leepel, Sastra, Puspitawati, & Bachtiar, 2012).

Regarding erythritol, this compound was shown to increase the effect of benzethonium chloride against *C. albicans,* leading to the assumption that erythritol can help to disperse biofilms, favoring the penetration of fungicidal agents and weakening the microbial bond to surfaces (Ichikawa, Yano, Fujita, Kashiwabara, & Nagao, 2008). However, the literature on this topic is scarce, pointing to the need for more comprehensive studies on the relationship between erythritol and this fungus.

A previous study showed that the association of F (500 ppm) and TMP on a biofilm of *S. mutans* and *C. albicans* was shown to promote alterations on the extracellular matrix and biofilm architecture compared with 1100 ppm F without TMP (Cavazana et al., 2019). Such effects were attributed to the interaction of these compounds, which directly acted on the inhibition of bacterial enzymes and the reduction of intra- and extra-cellular polysaccharides production (Hyun Koo, Sheng, Nguyen, & Marquis, 2006; Pandit, Kim, Jung, Chang, & Jeon, 2011). Based on this rationale, it could be hypothesized that the inhibitory effect for both *S. mutans* and *C. albicans* in the present study would result from the interaction (in different associations) of xylitol, erythritol, TMP, and/or F, through different mechanisms. Previous studies reported that these compounds act on cell metabolism, affecting the cytoplasm (Talattof et al., 2018), DNA and RNA (Janus et al., 2017), proteins and carbohydrates of the extracellular matrix (Cavazana et al., 2019), and adherence to surfaces (Leepel et al., 2012).

In general, our data point to a significantly higher effect of the experimental toothpaste on the growth inhibition of most of the microorganisms assessed. This supports the idea of simultaneous administration of polyols, polyphosphate, and F at a reduced concentration (200 ppm F) as an alternative to manage cariogenic biofilms, especially for young children, as it would reduce systemic fluoride exposure, while sustaining its mineralizing effects (from F and TMP) and enhancing the antimicrobial effects (from all the actives) compared with a conventional (1100 ppm F) toothpaste. However, any extrapolations to clinical conditions would be extremely premature given the preliminary nature of the data obtained, and limitations inherent to the study protocol. Future studies with biofilms, especially formed on dental substrates, could bring important data in this regard.

Conclusion

The association of TMP, F, xylitol, and erythritol was shown to be effective in reducing the growth of isolated cariogenic microorganisms under most of the conditions studied (*i.e.*, microorganisms and dilutions), being, therefore, a promising alternative to control biofilms related to dental caries.

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Tables

Table 1. Zone of inhibition (in mm) of toothpastes containing different active compounds, at 5 dilutions, on *Streptococcus mutans*

	TOOTHPASTE DILUTIONS (IN DEIONIZED WATER)				
GROUPS	D1	D2	D4	D8	D16
PLA	10.7 ± 0.3 Aa (11.8 – 13.6)	10.4 ± 0.4 Bab (9.4 – 11.5)	10.5 ± 0.8 Ba (8.7 – 12.4)	10.5 ± 0.5 Bab (9.3 – 11.6)	10.3 ± 0.5 Bab (9.1 – 11.6)
X	10.8 ± 0.6 Ab (9.2 – 12.3)	10.4 ± 0.5 Aab (9.0 – 11.7)	10.2 ± 0.4 Aa (9.2 – 11.2)	10.1 ± 0.3 Ab (9.4 – 10.8)	10.0 ± 0.3 Aab (9.5 – 10.7)
E	10.2 ± 0.2 Ab (9.8 – 10.6)	10.5 ± 0.5 Aab (9.2 – 11.6)	10.3 ± 0.4 Aa (9.1 – 11.4)	10.5 ± 0.5 Aab (9.4 – 11.5)	9.8 ± 0.2 Ab (9.3 – 10.1)
TMP	10.8 ± 0.2 Ab (10.3 – 11.1)	10.5 ± 0.5 Bab (9.3 – 11.7)	10.3 ± 0.4 Ba (8.6 – 11.9)	10.6 ± 0.4 Bab (9.4 – 11.7)	10.4 ± 0.3 Bab (9.4 – 11.0)
200F	10.2 ± 0.6 Ab (12.2 – 11.5)	12.2 ± 0.6 Bb (11.5 – 12.6)	11.7 ± 0.1 BCb (11.7 – 11.8)	11.7 ± 0.4 BCc (11.6 – 12.0)	11.9 ± 0.4 Cc (11.8 – 14.1)
1100F	11.1 ± 0.4 Ab (10.0 – 11.9)	11.2 ± 0.2 Aab (10.6 – 11.5)	11.1 ± 0.4 Aa (10.8 – 11.4)	11.6 ± 0.3 Aac (11.4 – 11.7)	11.3 ± 0.3 Aa (10.7 -11.9)
X+E	11.4 ± 0.8 Aab (9.3 – 13.3)	10.2 ± 0.5 Aa (8.9 – 11.4)	11 ± 0.5 Aa (9.9 – 12.0)	10.5 ± 0.5 Aab (9.4 – 11.5)	10.2 ± 0.2 Aab (9.7 – 10.5)
200F+TMP	21.1 ± 0.2 Ac (20.9 – 21.2)	20.3 ± 0.4 Bc (19.2 – 21.5)	19.9 ± 0.3 Bc (19.7 – 20.0)	19.5 ± 0.5 Bd (19.4 – 19.9)	19.2 ± 0.2 Bd (19.1 – 19.4)
200F+X+E	11.4 ± 0.4 Aab (10.3 – 12.4)	11.4 ± 0.3 Ab (11.2 – 12.6)	11.3 ± 0.4 Cb (11.1 – 11.5)	11.3 ± 0.3 Cc (11.0 – 11.4)	11.2 ± 0.3 Cc (11.1 – 11.3)
TMP+X+E	11.1 ± 0.6 Ab (9.5 – 12.6)	11.0 ± 0.5 Aab (9.8 – 12.1)	10.7 ± 0.5 Aa (9.5 – 11.8)	10.6 ± 0.5 Aab (9.4 – 11.7)	10.4 ± 0.6 Aab (8.8 – 12.0)
EXP	10.7 ± 0.4 Ab (9.3 – 12.0)	10.2 ± 0.2 Aa (9.3 – 11.0)	10.2 ± 0.6 Aa (9.9 – 11.1)	10.1 ± 0.4 Aab (10.0 – 10.4)	10.0 ± 0.5 Ab (10.0 – 10.5)

Results are presented as means ± SD (95% Confidence Intervals). Distinct upper-case letters indicate statistical significance for comparisons among dilutions within each group. Distinct lower-case letters indicate statistical significance for comparisons among treatments with each dilution (two-way ANOVA and Tukey's HSD test, $p < 0.05$; $n = 9$). PLA: Placebo, X: Xylitol (16%), E: Erythritol (4%), TMP: Sodium Trimetaphosphate (0.25%), 200F: 200 ppm Fluoride, 1100F: 1100 ppm Fluoride, XE: Xylitol (16%) + Erythritol (4%), 200F+TMP: 200 ppm Fluoride + Sodium Trimetaphosphate (0.25%), 200F+X+E: 200 ppm Fluoride + Xylitol (16%) + Erythritol (4%), TMP+X+E: Sodium Trimetaphosphate (0.25%) + Xylitol (16%) + Erythritol (4%), EXP: 200 ppm of Fluoride + Xylitol (16%) + Erythritol (4%) + Sodium Trimetaphosphate (0.25%).

Table 2. Zone of inhibition (in mm) of toothpastes containing different active compounds, at 5 dilutions, on *Lactobacillus casei*

	TOOTHPASTE DILUTIONS (IN DEIONIZED WATER)				
GROUPS	D1	D2	D4	D8	D16
PLA	13.4 ± 0.3 Aab (12.6 – 14.2)	12.0 ± 0.1 Ba (11.8 – 12.1)	12.0 ± 0.4 Bab (10.8 – 13.4)	11.2 ± 0.2 Bab (10.5 – 11.8)	6.5 ± 0.4 Ca (5.4 – 7.4)
X	15.4 ± 0.2 Ad (14.7 – 16.0)	12.9 ± 0.1 Bab (12.6 – 13.1)	12.0 ± 0.4 Babc (11.1 – 13.0)	11.9 ± 0.2 Bb (11.5 – 12.1)	6.5 ± 0.2 Ca (5.9 – 7.1)
E	13.1 ± 0.3 Aab (12.9 – 13.2)	13.4 ± 0.4 Ab (13.4 – 13.8)	11.4 ± 0.4 Ba (10.2 – 12.5)	11.6 ± 0.3 Bab (11.4 – 11.7)	11.8 ± 0.3 Bb (11.5 – 11.8)
TMP	12.8 ± 0.3 Abc (12.6 – 12.9)	12.6 ± 0.1 ABab (12.4 – 12.7)	13.2 ± 0.2 Ac (13.0 – 13.3)	11.5 ± 0.9 Bab (9.2 – 13.6)	6.5 ± 0.3 Ca (5.8 – 7.1)
200F	12.0 ± 0.1 Ac (11.5 – 12.3)	12.3 ± 0.5 Aab (11.1 – 13.5)	11.5 ± 0.5 Aa (11.0 – 11.6)	6.7 ± 0.2 Bc (6.2 – 7.1)	6.8 ± 0.2 Ba (6.3 – 7.1)
1100F	13.9 ± 0.2 Aa (13.4 – 14.4)	12.9 ± 0.2 ABab (12.7 – 13.0)	11.9 ± 0.6 Ba (11.0 – 12.7)	10.7 ± 0.8 Ca (8.8 – 12.6)	6.8 ± 0.2 Da (6.6 – 6.9)
X+E	13.2 ± 0.1 Aab (13.0 – 13.3)	13.2 ± 0.1 Ab (13.0 – 13.3)	11.9 ± 0.1 Ba (11.6 – 12.2)	10.8 ± 0.2 Bab (10.2 – 11.4)	6.1 ± 0.7 Ca (4.4 – 7.7)
200F+TMP	11.9 ± 0.1 Ac (11.8 – 12.1)	12.1 ± 0.2 Aa (11.9 – 12.2)	11.9 ± 0.3 Aa (11.0 – 12.6)	11.0 ± 0.2 ABab (10.8 – 11.1)	9.9 ± 0.2 Bc (9.7 – 10.0)
200F+X+E	13.9 ± 0.2 Aab (13.4 – 14.4)	13.4 ± 0.4 Bc (13.1 – 13.6)	13.3 ± 0.4 Ac (13.2 – 13.5)	11.5 ± 0.1 Cab (11.3 – 11.6)	6.2 ± 0.3 Da (5.2 – 7.1)
TMP+X+E	13.1 ± 0.2 Aab (12.9 – 13.2)	14.8 ± 0.3 Bd (14.5 – 14.8)	12.4 ± 0.9 Aabc (10.2 – 14.5)	12.2 ± 1.0 Bd (12.6 – 17.7)	6.5 ± 0.3 Ca (5.9 – 7.1)
EXP	14.2 ± 0.2 Aa (14.0 – 14.4)	13.5 ± 0.3 Bab (13.1 – 13.9)	13.1 ± 0.3 Bbc (13.0 – 13.2)	11.0 ± 0.2 Cab (10.8 – 11.1)	9.3 ± 0.4 Dc (9.1 – 9.5)

Results are presented as means ± SD (95% Confidence Intervals). Distinct upper-case letters indicate statistical significance for comparisons among dilutions within each group. Distinct lower-case letters indicate statistical significance for comparisons among treatments with each dilution (two-way ANOVA and Tukey's HSD test, $p < 0.05$; $n = 9$). PLA: Placebo, X: Xylitol (16%), E: Erythritol (4%), TMP: Sodium Trimetaphosphate (0.25%), 200F: 200 ppm Fluoride, 1100F: 1100 ppm Fluoride, XE: Xylitol (16%) + Erythritol (4%), 200F+TMP: 200 ppm Fluoride + Sodium Trimetaphosphate (0.25%), 200F+X+E: 200 ppm Fluoride + Xylitol (16%) + Erythritol (4%), TMP+X+E: Sodium Trimetaphosphate (0.25%) + Xylitol (16%) + Erythritol (4%), EXP: 200 ppm of Fluoride + Xylitol (16%) + Erythritol (4%) + Sodium Trimetaphosphate (0.25%).

Table 3. Zone of inhibition (in mm) of toothpastes containing different active compounds, at 5 dilutions, on *Actinomyces israelii*

TOOTHPASTE DILUTIONS (IN DEIONIZED WATER)					
GROUPS	D1	D2	D4	D8	D16
PLA	5.9 ± 0.1 Aa (5.8 – 6.1)	5.6 ± 0.2 Aa (5.3 – 6.0)	5.8 ± 0.3 Aa (5.2 – 6.5)	6.1 ± 0.2 Aa (5.6 – 6.4)	6.1 ± 0.1 Aa (5.8 – 6.4)
X	5.9 ± 0.2 Aa (5.5 – 6.3)	5.9 ± 0.1 Aa (5.7 – 6.1)	6.1 ± 0.2 Aa (5.7 – 6.5)	5.9 ± 0.1 Aa (5.6 – 6.2)	6.0 ± 0.3 Aab (5.1 – 6.9)
E	6.2 ± 0.1 Aa (6.2 – 6.2)	6.1 ± 0.2 Aa (5.7 – 6.4)	6.0 ± 0.2 Aa (5.7 – 6.4)	5.9 ± 0.1 Aa (5.6 – 6.2)	6.0 ± 0.1 Aab (5.5 – 6.3)
TMP	5.9 ± 0.2 Aa (5.8 – 6.3)	6.0 ± 0.4 Aa (5.6 – 6.4)	5.9 ± 0.3 Aa (5.7 – 6.0)	6.0 ± 0.2 Aa (5.4 – 6.6)	5.9 ± 0.3 Aab (5.6 – 6.1)
200F	6.2 ± 0.1 Aa (6.0 – 6.3)	5.8 ± 0.1 Aa (5.7 – 6.0)	5.7 ± 0.1 Aa (5.2 – 6.1)	6.7 ± 0.2 Bb (6.2 – 7.1)	6.7 ± 0.4 Bc (6.2 – 6.9)
1100F	5.7 ± 0.3 Aa (5.5 – 6.0)	6.0 ± 0.3 Aa (5.8 – 6.3)	5.8 ± 0.2 Aa (5.4 – 6.2)	5.9 ± 0.2 Aa (5.3 – 6.6)	6.0 ± 0.2 Aab (6.0 – 6.2)
X+E	6.0 ± 0.1 Aa (5.8 – 6.1)	5.7 ± 0.2 Aa (5.2 – 6.2)	5.8 ± 0.4 Aa (5.6 – 6.1)	5.7 ± 0.3 Aa (5.4 – 6.0)	5.9 ± 0.2 Aab (5.5 – 6.4)
200F+TMP	5.7 ± 0.2 Aa (5.2 – 6.3)	5.7 ± 0.2 Aa (5.2 – 6.2)	5.6 ± 0.2 Aa (5.2 – 6.1)	5.8 ± 0.3 Aa (5.3 – 6.3)	5.5 ± 0.4 Ab (5.1 – 6.0)
200F+X+E	5.7 ± 0.2 Aa (5.3 – 6.2)	5.8 ± 0.3 Aa (5.0 – 6.6)	5.7 ± 0.3 Aa (5.5 – 6.0)	5.9 ± 0.1 Aa (5.7 – 6.0)	5.1 ± 0.2 Aab (5.6 – 6.1)
TMP+X+E	6.0 ± 0.2 Aa (5.8 – 6.1)	5.9 ± 0.1 Aa (5.7 – 6.2)	5.8 ± 0.3 Aa (5.6 – 6.0)	5.9 ± 0.3 Aa (5.7 – 6.0)	5.9 ± 0.3 Aab (5.7 – 6.0)
EXP	5.8 ± 0.1 Aa (5.6 – 5.9)	6.0 ± 0.3 Aa (5.6 – 6.4)	5.9 ± 0.1 Aa (5.8 – 6.1)	5.9 ± 0.3 Aa (5.5 – 6.3)	6.1 ± 0.3 Aa (5.8 – 6.4)

Results are presented as means ± SD (95% Confidence Intervals). Distinct upper-case letters indicate statistical significance for comparisons among dilutions within each group. Distinct lower-case letters indicate statistical significance for comparisons among treatments with each dilution (two-way ANOVA and Tukey's HSD test, $p < 0.05$; $n = 9$). PLA: Placebo, X: Xylitol (16%), E: Erythritol (4%), TMP: Sodium Trimetaphosphate (0.25%), 200F: 200 ppm Fluoride, 1100F: 1100 ppm Fluoride, XE: Xylitol (16%) + Erythritol (4%), 200F+TMP: 200 ppm Fluoride + Sodium Trimetaphosphate (0.25%), 200F+X+E: 200 ppm Fluoride + Xylitol (16%) + Erythritol (4%), TMP+X+E: Sodium Trimetaphosphate (0.25%) + Xylitol (16%) + Erythritol (4%), EXP: 200 ppm of Fluoride + Xylitol (16%) + Erythritol (4%) + Sodium Trimetaphosphate (0.25%).

Table 4. Zone of inhibition (in mm) of toothpastes containing different active compounds, at 5 dilutions, on *Candida albicans*

	TOOTHPASTE DILUTIONS (IN DEIONIZED WATER)				
GROUPS	D1	D2	D4	D8	D16
PLA	5.6 ± 0.2 Aa (4.9 – 6.4)	5.5 ± 0.1 Aa (5.1 – 5.9)	5.6 ± 0.3 Aa (5.3 – 5.8)	5.8 ± 0.7 Aa (5.7 – 6.0)	5.6 ± 0.3 Aab (5.5 – 5.8)
X	5.7 ± 0.1 Aa (5.6 – 5.9)	5.7 ± 0.3 Aa (5.3 – 6.2)	5.7 ± 0.3 Aa (4.9 – 6.4)	5.9 ± 0.5 Aa (5.7 – 6.8)	5.4 ± 0.4 Aa (5.2 – 5.5)
E	5.9 ± 0.3 Aa (5.2 – 6.5)	5.8 ± 0.3 Aa (5.4 – 6.2)	5.9 ± 0.5 Aab (5.2 – 6.7)	5.8 ± 0.3 Aa (5.0 – 6.6)	5.9 ± 0.3 Aab (5.2 – 6.7)
TMP	6.2 ± 0.2 Aab (5.5 – 6.9)	5.1 ± 0.1 ABa (5.1 – 6.0)	5.1 ± 0.2 ABab (5.0 – 6.1)	5.4 ± 0.2 Ba (5.2 – 6.5)	5.7 ± 0.5 ABab (5.3 – 6.1)
200F	5.8 ± 0.1 Aa (5.5 – 6.1)	5.5 ± 0.1 Aa (5.1 – 5.9)	5.6 ± 0.9 Aa (5.3 – 5.9)	5.8 ± 0.1 Aa (5.4 – 6.2)	5.6 ± 0.7 Aab (5.4 – 5.7)
1100F	6.1 ± 0.2 Aab (5.5 – 6.7)	5.9 ± 0.2 Aab (5.7 – 6.2)	6.1 ± 0.3 Aab (5.4 – 6.8)	5.8 ± 0.2 Aa (5.4 – 6.3)	5.8 ± 0.1 Aab (5.5 – 6.0)
X+E	6.0 ± 0.5 Aab (5.8 – 6.3)	5.1 ± 0.3 Aa (5.1 – 6.0)	5.1 ± 0.2 Aa (5.1 – 5.4)	5.1 ± 0.3 Aa (5.0 – 5.7)	5.0 ± 0.9 Aab (5.0 – 5.4)
200F+TMP	5.8 ± 0.1 Aa (5.5 – 6.1)	5.5 ± 0.4 Aa (5.1 – 5.9)	5.6 ± 0.2 Aa (5.3 – 5.9)	5.8 ± 0.1 Aa (5.4 – 6.2)	5.6 ± 0.7 Aab (5.4 – 5.7)
200F+X+E	6.6 ± 0.1 ABb (6.4 – 6.7)	6.6 ± 0.2 Bb (6.3 – 6.9)	5.9 ± 0.4 ACab (5.6 – 6.2)	5.7 ± 0.3 Ca (5.3 – 6.1)	5.9 ± 0.2 ACab (5.3 – 6.5)
TMP+X+E	5.7 ± 0.2 Aa (5.4 – 5.9)	5.7 ± 0.2 Aa (5.2 – 6.2)	5.8 ± 0.2 Aa (5.5 – 6.0)	5.8 ± 0.1 Aa (5.5 – 6.5)	5.7 ± 0.7 Aab (5.2 – 6.2)
EXP	7.9 ± 0.4 Ac (6.7 – 9.1)	7.6 ± 0.3 Ac (6.6 – 8.5)	6.6 ± 0.2 Bb (6.1 – 7.1)	6.0 ± 0.7 Ba (5.4 – 6.7)	6.0 ± 0.1 Bb (5.9 – 6.5)

Results are presented as means ± SD (95% Confidence Intervals). Distinct upper-case letters indicate statistical significance for comparisons among dilutions within each group. Distinct lower-case letters indicate statistical significance for comparisons among treatments with each dilution (two-way ANOVA and Tukey's HSD test, $p < 0.05$; $n = 9$). PLA: Placebo, X: Xylitol (16%), E: Erythritol (4%), TMP: Sodium Trimetaphosphate (0.25%), 200F: 200 ppm Fluoride, 1100F: 1100 ppm Fluoride, XE: Xylitol (16%) + Erythritol (4%), 200F+TMP: 200 ppm Fluoride + Sodium Trimetaphosphate (0.25%), 200F+X+E: 200 ppm Fluoride + Xylitol (16%) + Erythritol (4%), TMP+X+E: Sodium Trimetaphosphate (0.25%) + Xylitol (16%) + Erythritol (4%), EXP: 200 ppm of Fluoride + Xylitol (16%) + Erythritol (4%) + Sodium Trimetaphosphate (0.25%).

CAPÍTULO 2

Evaluation of solutions containing fluoride, sodium trimetaphosphate, xylitol and erythritol on dual-species biofilms

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Este artigo segue as normas do periódico Biofouling (Anexo C).

Evaluation of solutions containing fluoride, sodium trimetaphosphate, xylitol and erythritol on dual-species biofilms

Abstract

The aim of this study was to assess the effect solutions containing fluoride, sodium trimetaphosphate (TMP), xylitol and erythritol on dual-species biofilms of *S. mutans* and *C. albicans*. Biofilms were grown in the continuous presence these actives alone or in different associations. Quantification of Colony-Forming unity (CFU), metabolic activity, biomass, and extracellular matrix components were evaluated. CFU counts were mostly affected by F, while biomass and metabolic activity were more susceptible to TMP. A synergistic effect of these actives was observed. As for the polyols, their effect was more pronounced on the carbohydrate content of the extracellular matrix, with lesser or no action on the other variables. Overall, the association of the four actives led to increased antibiofilm effects. The dual-species biofilms analyzed were mostly affected by F and/or TMP, with little effects of the polyols administered alone. Co-administration of the four actives was effec4tive in reducing biofilm's virulence.

Keywords: Biofilms, *Streptococcus mutans*, *Candida albicans*, phosphate, sugar alcohols, fluoride

Word count: 3.835

The authors report there are no competing interests to declare

Introduction

Dental caries is considered a dynamic disease, biofilm-mediated and diet modulated, resulting in loss of the mineral dental tissues (Machiulskiene et al., 2020). One commensal member of all microorganisms related onset of this disease is *Streptococcus mutans*, which is found in caries lesions of both adults and children (Menon et al., 2022). Besides, *Candida albicans* is a commensal fungi found in 96% of children with dental caries (Raja, Hannan, & Ali, 2010), and oral cavities already colonized by this fungus may have nearly five times a greater risk of developing early childhood caries (Xiao et al., 2016). Furthermore, *S. mutans* can produce anchored proteins that allow and facilitate the binding to *C. albicans*, leading to a more virulent environment in the biofilm (Bamford et al., 2009).

Fluoride (F) products play an important role in preventing, controlling, and arresting dental caries. Among the most used formulations, there is strong evidence on the clinical efficacy of toothpastes containing ~1100 ppm F or above in reducing the progression of the disease (Sampaio et al., 2020; Walsh et al., 2019), with a lower number of trials attesting the efficacy of formulations with lower F content. However, a there is a strong body of evidence from *in vitro*, *in situ*, and clinical studies showing that the addition of sodium trimetaphosphate (TMP) to low-F toothpastes promotes synergistic effects on de- and re-mineralization, and on the progression of caries lesions in children (Freire et al., 2016; Takeshita et al., 2016, 2015). Moreover, TMP presents effects over dual-species biofilms of *S. mutans* and *C. albicans*, acting directly on the metabolic activity and production of biofilm biomass, in addition to effects on protein, carbohydrate and DNA content of the extracellular matrix (Cavazana et al., 2019).

In addition to the actives above, natural products such as xylitol and erythritol have demonstrated marked effects on biofilms (Cannon et al., 2020; Loimaranta, Mazurel, Deng, & Söderling, 2020). Both polyols have been shown to decrease biofilm acidogenicity and volume, and to inhibit the growth of *S. mutans*. The effects of these polyols over the extracellular matrix have also shown to decrease the microorganism's adherence to enamel surface and to decrease the expression of genes involved in the metabolism of sucrose (Park et al., 2014).

Given the different mechanisms of action of F, TMP, xylitol and erythritol on cariogenic biofilms, it would be possible that the association of these actives could

increase the antibiofilm effects of topically-applied formulations. Thus, this study aimed to assess the effects of F, TMP, xylitol or erythritol, alone or in different combinations, on dual-species biofilms of *S. mutans* and *C. albicans*, using different biofilm quantification assays (cultivable cells, total biomass, metabolic activity, and extracellular matrix composition). The study's null hypothesis was that the effects of the actives alone would not be significantly different from those observed by different associations on the variables analyzed.

Materials and methods

Artificial saliva

The culture medium used for biofilm formation was sucrose-containing artificial saliva (AS), and its composition for 1.0 l of deionized water was based on the protocol described by Lamfon et al. (2003) with modifications: 4 g of sucrose (Sigma-Aldrich, St Louis, MO, USA), 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 the test solutions

The solutions were prepared by weighing and diluting the following compounds: xylitol (Sigma-Aldrich), erythritol (Sigma-Aldrich), trisodium trimetaphosphate (Sigma-Aldrich), or sodium fluoride (NaF, Sigma-Aldrich), alone or in different associations, in order to achieve final concentrations of 4.8%, 1.2%, 0.075%, and 60 ppm F, respectively. The experimental groups in this study were: Pure AS (negative control = "NC"), NaF 330 ppm F (positive control – "330F"), Xylitol 4.8% ("X"), Erythritol 1.2% ("E"), Sodium trimetaphosphate 0.075% ("TMP"), NaF 60 ppm F ("60F"), "X+E", "60F+TMP", "TMP+X+E", "60F+X+E", and experimental solution containing 60F+TMP+X+E ("EXP"). All compounds were filter-sterilised to obtain an aseptic condition. The concentrations were adopted to achieve 30% of the initial concentrations used in a previous study, administered as toothpastes (Marcato et al., 2021).

Strains and growth conditions

Two strains from the American Type Culture Collection (ATCC) were included 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 of Sabouraud Dextrose Broth (Difco) and aerobically incubated overnight at 120 rpm and 37 ºC. Simultaneously, *S. mutans* colonies grown on Brain Heart Infusion agar (BHI Agar; Difco) were suspended in 10 ml of BHI broth (Difco) and statically incubated overnight in 5% CO_2 at 37 ºC. Afterwards, fungal and bacterial cells were recovered by centrifugation (8,000 rpm, 5 min), and the cell pellets washed twice with 10 ml of 0.85% NaCl. The number of *C. albicans* cells was adjusted to $1x10^7$ cells ml^{-1} in AS using a Neubauer counting chamber, while the number of bacterial cells was spectrophotometrically (640 nm) adjusted to $1x10^8$ cells ml^{-1}.

Biofilm formation and growth in the presence of solutions

Dual-species biofilms were formed in flat-bottom 96-well microtiter plates (Costar, Tewksbury, USA). For this, 100 µL of each microbial suspension ($2x10^7$ cells ml^{-1} for *C. albicans*, $2x10^8$ cells ml^{-1} for *S. mutans*) were added to the wells and the plates were incubated in 5% CO_2 at 37 ºC for 2 h. Next, AS was removed, and the wells were washed once with 0.85% NaCl. Each test solution was added to AS to achieve a final solution of 4 mL. These compounds were then pipetted in the walls containing adhered cells, and the plates were incubated for 24 h in 5% CO_2 at 37 ºC in order to allow biofilm formation (Vieira et al., 2019). The medium was renewed every 24 h by removing 100 ml and adding an equal volume of fresh test solution. After completing 96 h biofilm formation, the test solutions were removed from the wells, and the resulting biofilms were washed once with 0.85% NaCl to eliminate planktonic cells.

Biofilm quantification assays

Cultivable cells

The number of cultivable cells was assessed by enumeration of Colony-Forming Units (CFUs), as previously detailed (Fernandes et al., 2016). 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 µL ml^{-1}

amphotericin B (Sigma-Aldrich). Agar plates were incubated for 24–48 h at 37 ºC, 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 (Monteiro et al., 2011). Biofilms were fixed for 15 min at room temperature with 99% methanol (Sigma-Aldrich), stained for 5min with 1% CV (Sigma-Aldrich), and de-stained through exposure 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 metabolic activity of biofilm cells was performed by the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma-Aldrich) reduction method (Fernandes et al., 2016). In summary, XTT and phenazine methosulphate (Sigma-Aldrich) solutions were combined and pipetted into the wells. The microliter plates were incubated (37 ºC, 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 grown in the six-well plates (Costar) containing 4 ml of the microbial suspension, as described above. After the 96 h with solutions, the biofilms were resuspended in 0.85% NaCl, scraped from the wells, and the liquid phase of the extracellular matrix was extracted by sonication (for 30 s at 30W), as detailed elsewhere (S. Silva et al., 2009). The bicinchoninic acid method (Kit BCA; Sigma-Aldrich) was performed for protein determination of the extracellular matrix, using bovine serum albumin as the standard (S. Silva et al., 2009), while the carbohydrate content was measured using the method devised by Dubois et al. (1956), with glucose as the standard. For DNA content, a volume of 1.5 µl of the liquid phase of the extracellular matrix was spectrophotometrically analyzed (at 260 and 280 nm) in a Nanodrop Spectrophotometer (EONC Spectrophotometer of EONC, Biotek, Winooski, VT, USA) (Monteiro et al., 2013). Protein, carbohydrate and DNA values were expressed as mg g^{-1} dry weight of biofilm.

Statistical analysis

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

Results

Regarding the number of viable cells of *S. mutans*, all test groups had significantly lower values compared to the NC, except for TMP alone (Figure 1a). Overall, the largest reductions were observed for 330F, followed by 60F+TMP, EXP and 60F, with a synergistic effect observed between TMP and 60F. For *C. albicans*, all test groups had significantly lower numbers of viable cells compared to the NC, with no clearly defined trend among groups containing the actives (Figure 2b).

Solutions containing TMP, TMP+X+E, or 60F+TMP promoted significantly larger reductions in the total biofilm mass compared to the other groups (Figure 1c). On the other hand, solutions containing X, E, or X+E did not promote significant reductions compared to the NC. A similar trend was observed for the metabolic activity of the biofilms assessed (Figure 1d).

All test solutions were able to reduce the protein and carbohydrate contents of the extracellular matrix of the biofilms compared to the NC (Table 1), with the largest effects seen for 330F and 60F (dose-response), TMP and 60F+TMP. As for DNA content, a similar pattern was observed, except that the polyols alone or associated with 60F or TMP did not promote significant reductions compared to the NC.

Discussion

The high consumption of fermentable sugars in the diet, allied with the absence of proper hygiene of the oral cavity, create favorable conditions for the growth and development of cariogenic biofilms, especially at stagnation sites (Machiulskiene et al., 2020). To overcome this issue, consolidated F-based strategies have been tested in association with new preventive/therapeutic agents, which have been shown to promote significant effects on enamel de- and re-mineralization, as well as in reducing biofilm formation and, consequently, dental caries (Pessan et al., 2021). Within this context, the present study aimed to evaluate the effects of solutions containing F, TMP, X and E, alone or in different combinations, demonstrating that most of the associations affected several variables related to dual-species cariogenic biofilms of *S. mutans* and *C. albicans*, thus leading to partial rejection of the null hypothesis.

Among the more than 700 different species of microbes that inhabit the oral cavity, *S. mutans* is one of the main cariogenic pathogens, which is related to the onset of caries lesions (Kilian et al., 2016; Matsumoto-Nakano, 2018). The present findings showed that the major effect on *S. mutans* CFU counts was promoted by F, in a dose-response manner (Figure 1a). Such effects were somehow expected considering the well-known bacteriostatic action of F on this bacterium (Buzalaf et al., 2011), what might be potentiated by the constant presence of this active during initial biofilm formation and growth. Interestingly, such bacteriostatic effects seem to have affected all the other variables analyzed, despite at a lesser extent and not following a dose-response trend. As for TMP, it was noteworthy that despite this polyphosphate promoted no reduction in *S. mutans* CFU counts when administered alone (compared with the negative control), it potentiated the effects of F. In fact, similar results were found by Cavazana et al. (2019) using the same biofilm model, but administering the actives at higher concentrations and in short-term exposure times (instead of its constant presence during biofilm formation).

The synergism between the actives F and TMP has been extensively reported for enamel de- and re-mineralization when administered as solutions, toothpastes, mouthwashes, gels and varnishes (Cavazana et al., 2019; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014; Freire et al., 2016; Michele M. Manarelli et al., 2017; Takeshita et al., 2016, 2015). Collectively, these studies demonstrate that

TMP's mechanism of action is related to its ability to adsorb onto the enamel surface, what hinders acid diffusion and decreases enamel demineralization. In our study, however, the absence of tooth enamel in the model suggests that the positive effects of F + TMP on the biofilms assessed might mostly be attributed to the inhibition of acidic niche formation and cell adhesion and aggregation due to the action of TMP (Flemming & Wingender, 2010). This hypothesis is supported by a previous study using the same dual-species model as in the present work, showing an enhanced buffering effect of F + TMP in comparison with the actives alone (Cavazana et al., 2020).

The aforementioned synergism of F + TMP on CFU counts of *S. mutans* were, in general, reflected on all the other parameters assessed, including biofilm biomass, metabolic activity and, to a lesser extent, on the protein content of the extracellular matrix. Interestingly, however, the highest effects on biofilm biomass and metabolic activity were not promoted by F, but instead by TMP, and this polyphosphate was shown to potentiate the effects also of the polyols on both variables analyzed (Figure 1c and 1d). Regarding the extracellular matrix, TMP and F (administered alone or in association) also promoted significant reductions in protein, carbohydrate and DNA contents. Following a similar rationale to that described for F, it seems plausible that the effects of TMP on biofilm biomass and metabolic activity resulted in lower contents of the extracellular matrix components, with a synergistic effect with F on its protein content (Table 1). These findings are in line with previous data obtained by the use of F + TMP as treatment solutions, which demonstrated significant reductions in total biomass values, metabolic activity, and extracellular matrix components in mixed biofilms of *S. mutans* and *C. albicans* (Cavazana et al., 2019). Also, the administration of toothpastes containing these actives on polymicrobial biofilms formed *in situ* resulted in lower production of extracellular polysaccharides compared with F alone (Takeshita et al., 2015). A possible explanation for these reductions is related to a slight chelating ability of TMP (Vaara, 1992), which allows its binding to the cell wall of gram-positive bacteria, specifically through Ca^{+} and Mg^{+} ions, thus leading to changes in bacterial metabolism (Klompmaker, Kohl, Fasel, & Mayer, 2017; H. Koo, Falsetta, & Klein, 2013; Lee, Hartman, Stahr, Olson, & Williams, 1994). This reduction may also be associated with the bioavailability of nutrients in the culture medium, since possible interactions between TMP and ions

present in the medium may occur, causing a reduction in biofilm metabolism (Cavazana et al., 2019; Lee et al., 1994).

As for xylitol and erythritol, the data obtained analyzed together point out to very modest effects of these polyols on the biofilms compared with the negative control. Although at first glance the trend observed was not expected, the sucrose content in the culture medium and the maturation stage of the biofilms likely played major roles on the results obtained. Given that sucrose was not administered as pulses (to mimic cariogenic challenges), but instead it was constantly present in the culture medium, bacteria would predominantly metabolize fructose and glucose (from sucrose) over sugar-alcohols, so it is seems unlikely that the polyols would be metabolized in the presence of hexoses (Chan et al., 2020; Zeng, Chen, & Burne, 2018). Similar results have been previously reported for single- and dual-species biofilms of *S. mutans* and *C. albicans* at mature formation stages (Eskandarian et al., 2017; Giertsen, Arthur, & Guggenheim, 2011). Conversely, biofilms at early stages (8h-24h) were shown to more susceptible to treatment with xylitol and erythritol compared with mature biofilms (Kościelniak et al., 2019; Loimaranta et al., 2020). Therefore, the time for biofilm formation in the present study (96 h) might also have influenced the results obtained concerning the effect of xylitol and erythritol.

Despite the small effects of the polyols on CFU counts, biofilm biomass, and on protein and DNA contents of the extracellular matrix, these actives promoted reductions of ~30% in the carbohydrate concentrations of the biofilms (Table 1). This is important from a clinical perspective, as this parameter is directly related with the biofilms virulence (Klein et al., 2015). Data on the metabolic activity also deserve comment, since the results from biofilms treated with the polyols were as high as those observed for the negative control. Analyzed together, these two parameters are in line with the well-known futile cycle of the polyols (E. Söderling et al., 2011), given that the presence of the sugar alcohols led to highly active biofilms (also resulting in high biofilm biomass), but significantly lower reserve of extracellular carbohydrates in the biofilm matrix.

The negligible effect of the actives over *C. albicans* cells (Figure 1b) must be addressed. Treatment with F and/or TMP has already been shown to be ineffective on this fungus (Cavazana et al., 2019, 2020), and the effects of polyols have usually been described for bacteria, so that the trend observed was not necessarily unexpected. It should be noted that *C. albicans* was used in the present study

because this dual-species biofilm model (*i.e.*, *S. mutans* and *C. albicans*) was previously validated as a cariogenic model of relatively low complexity, able to reproduce the pH changes following sucrose exposure similarly to what is observed *in vivo* (Cavazana et al., 2020). This model has successfully been used to assess the effects of F, alone or associated with polyphosphates, as well as antimicrobial drugs (Cavazana et al. 2019; Cavazana et al. 2020). However, this model has not been previously used to assess the effects of polyols, so that a few aspects deserve comment, especially the complexity of this fungus and the considerable size of the microorganism structure compared to prokaryotic cells (Fernandes et al., 2016). Moreover, when sucrose is present in the medium, a higher amount of hyphae cover the biofilm, what leads to a denser structure that hinders the access of actives to act over the biofilm cells (Barbosa et al., 2016; Chan et al., 2020; Fernandes et al., 2016). Also, *S. mutans* seems to stimulate the growth, and consequently increase the amount of *C. albicans* cells in the biofilm, making it more difficult for the active compounds to affect the biofilm cells (Cavazana et al., 2019; Fernandes et al., 2016). Considering that polymicrobial biofilms formed *in vivo* may present a less dense structure than that observed for the dual-species model used in the present study, our results must be interpreted in light with these considerations.

Finally, despite the apparent low effect of the polyols on some of the variables analyzed, it must be emphasized that the reductions in CFU counts, biofilm biomass, and protein and carbohydrate were significant when compared with the negative control. Most importantly, the association of the polyols with the other two actives (*i.e.*, experimental group) resulted in similar effects to those observed for the positive control (330F) regarding biofilm biomass and extracellular matrix components. This is paramount from a clinical standpoint, especially considering the effects of the polyols on enamel de- and re-mineralization (Cardoso et al., 2014; Marcato et al., 2021; V. M. Silva, Massaro, Buzalaf, Janson, & Garib, 2021), ought to their ability to form complexes with calcium ions and induce remineralization of deeper layers of enamel lesions (De Cock et al., 2016; Kauko K. Mäkinen, 2010). Thus, despite the limitations of *in vitro* biofilm models, the present results could have important implications on caries dynamics, as the simultaneous administration of the four actives could be effective on enamel de-/re-mineralization and on biofilm's virulence.

In conclusion, the dual-species biofilms analyzed were mostly affected by fluoride and/or TMP, with little effects of the polyols when administered alone.

However, co-administration of the four actives was effective in reducing biofilm's virulence. Future studies using more complex biofilm models, especially formed on mineralized dental substrates, could provide additional data on the actual effects of the actives alone and/or associated.

The authors report there are no competing interests to declare.

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Tables

Table 1. Mean (standard deviation) of protein, carbohydrate and DNA of dual-species biofilms obtained after treatment with different concentrations of fluoride, sodium trimetaphosphate, xylitol or erythritol, alone or in different associations

Matrix component (mg g^{-1} of biofilm dry weight)	**Groups**										
	NC	60F	60F+TMP	330F	EXP	TMP	60F+X+E	TMP+X+E	X	E	X+E
Proteins	31.37^{A} (1.97)	24.75^{B} (1.14)	22.57^{C} (1.76)	20.71^{D} (1.77)	23.62^{BC} (1.31)	$22.15D^{D}$ (1.80)	24.81^{B} (1.71)	27.18^{E} (1.60)	28.08^{E} (1.81)	27.95^{E} (1.00)	28.57^{E} (1.20)
Carbohydrates	556.4^{A} (28.6)	243.0^{BC} (16.1)	239.0^{CDE} (15.9)	210.5^{CFG} (19.5)	246.9^{BDG} (25.7)	209.4^{EF} (14.5)	254.7^{BD} (23.0)	363.2^{H} (24.3)	405.6^{I} (11.7)	365.1^{H} (22.1)	413.2^{I} (23.9)
DNA	14.5^{A} (0.67)	12.7^{BC} (0.42)	13.0^{BD} (0.39)	9.6^{B} (0.55)	12.8^{BE} (0.43)	10.7^{BF} (0.34)	13.2^{ACDEF} (0.31)	13.6^{ACDEF} (0.39)	14.0^{AD} (0.24)	14.2^{A} (0.54)	14.0^{ADE} (0.33)

Different upper-case letters symbolize statistical differences among the groups ($p<0.05$) (n=9). NC: negative control (untreated biofilms), 60F: 60 ppm Sodium Fluoride, 60F+TMP: 60 ppm Sodium Fluoride + 0.075% Sodium trimetaphosphate, 330F: 330 ppm Sodium Fluoride, EXP: 4.8% Xylitol + 1.2% Erythritol + 60 ppm Sodium Fluoride + 0.075% Sodium trimetaphosphate, TMP: 0.075% Sodium trimetaphosphate, 60F+X+E: 60 ppm Sodium Fluoride + 4.8% Xylitol + 1.2% Erythritol, TMP+X+E: 0.075% Sodium trimetaphosphate + 4.8% Xylitol + 1.2% Erythritol, X: 4.8% Xylitol, E: 1.2% Erythritol, X+E: 4.8% Xylitol + 1.2% Erythritol.

Figures

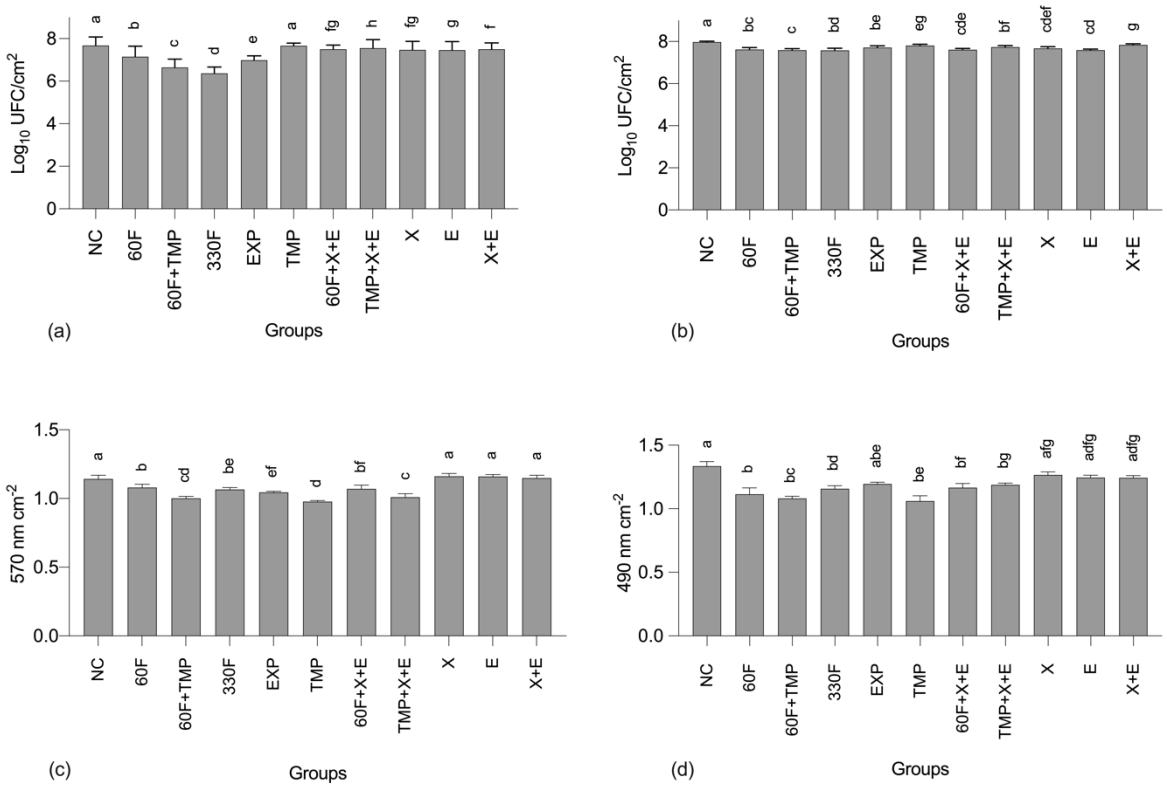

Figure 1. Logarithm of colony-forming units cm^{-2} for *S. mutans* (a) and *C. albicans* (b), and absorbance values cm^{-2} obtained for the total biomass (c) and metabolic activity (d) quantification assays. Error bars denote the SD of the means. Different lowercase letters symbolize statistical differences among the groups ($p<0.05$, $n=9$). NC: negative control (untreated biofilms), “60F” = 60 ppm F; “330F” = 330 ppm F; “X” = 4.8% Xylitol; “E” = 1.2% Erythritol.

CAPÍTULO 3

Assessment of the effects of fluoride, sodium trimetaphosphate, xylitol and/or erythritol on saliva-derived microcosm biofilms

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Este artigo segue as normas do periódico Archives of Oral Biology (Anexo D)

Abstract

Objective: This study evaluated the effects fluoride (F), sodium trimetaphosphate (TMP), xylitol (X) and/or erythritol (E) on the growth of saliva-derived microcosm biofilms.

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 0,013%, 0.075% TMP, 4.8% X and 1.2% E combined or not, were constantly present in the culture medium. In addition, 330 ppm F alone (330F) and McBain medium without any compound were also tested as positive and negative controls (NC), respectively. After 96 h, the biofilms were plated on anaerobic blood agar or sucrose agar bacitracin for total and *S. mutans* Colony-Forming Unity counting, respectively. Biofilms' lactic acid production was analyzed spectrophotometrically. Data were submitted to ANOVA or Kruskal-Wallis' tests, followed by Student-Newman-Keuls' test ($p<0.05$; $n=9$).

Results: 60F+TMP 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 330F and NC. The experimental group (containing all actives) also promoted interesting decreases over the parameters analysed.

Conclusions: The association of F+TMP and the experimental group reduced total and *S. mutans* CFU counts, as well as lactic acid production by saliva-derived microcosm biofilms.

Keywords: biofilms, fluorides, phosphates, sugar alcohols

Introduction

Robust evidence has demonstrated that fluoride (F) reduces the incidence and prevalence of dental caries, which is used for caries prevention and control in vehicles for self- and professional-application (Buzalaf, Pessan, Marques Honório, & Ten Cate, 2011; Walsh, Worthington, Glenny, Marinho, & Jeroncic, 2019). Nonetheless, the limited action of this ion has prompted to the search for strategies to enhance the effects of F-containing products, including polyols- and phosphate-based agents. Sodium trimetaphosphate (TMP) is an inorganic phosphate that presents promising effects on biofilm formation and on enamel de- and –re-mineralization (Cavazana et al., 2019; Takeshita, Danelon, Castro, Sassaki, & Delbem, 2015). In toothpastes, the association of F at low concentrations (i.e., 500 ppm F) and TMP promoted similar or superior effects than those observed for a conventional formulation (1100 ppm F) on enamel demineralization *in vitro* and *in situ*, and on caries progression *in vivo* (Freire et al., 2016; Takeshita, Danelon, Castro, Cunha, & Delbem, 2016; Takeshita et al., 2015).

Other active agents, such as xylitol and erythritol (polyols), have shown to have effects on the oral microbiome and on dental hard tissues (Marcato et al., 2021; Nayak, Nayak, & Khandelwal, 2014; Siqueira et al., 2021; E. Söderling & Pienihäkkinen, 2022). Xylitol a five-carbon polyalcohol sugar, that, is able to promote reductions in the number of the *Streptococcus mutans* (*S. mutans*) cells, lactic acid production and enamel remineralization (Cannon et al., 2020; Kõljalg, Smidt, Chakrabarti, Bosscher, & Mändar, 2020; Loimaranta, Mazurel, Deng, & Söderling, 2020; E. M. Söderling & Hietala-Lenkkeri, 2010). As for erythritol, it is a four-carbon polyalcohol sugar that seems to act in a similar effect to that of xylitol, demonstrating marked results on the reduction of *S. mutans* cells and promoting weakening effects over the microorganism adherence to the dental surfaces (De Cock et al., 2016; Hashino et al., 2013; Kõljalg et al., 2020).

Despite the substantial scientific evidence observed in *in vitro* biofilm studies assessing the effects of TMP or polyols, most of these studies were conducted with mono- or -dual-species biofilms formed in static models (Cannon et al., 2020; Cavazana et al., 2019, 2020; Kõljalg et al., 2020; Loimaranta et al., 2020), which do not reproduce the microbial complexity of the oral environment. In this sense, the use of microcosm biofilms *in vitro*, formed in a high-throughput model, allows for the

active growth of polymicrobial biofilms (Exterkate, Crielaard, & Ten Cate, 2010). Furthermore, given the different mechanisms by which F, TMP and polyols may affect cariogenic biofilms, it would be possible that the coadministration of these actives could increase the overall antibiofilm effect. The evidence to endorse such a hypothesis, however, is scarce.

Therefore, the aim of this study was to investigate the effects of solutions containing F, TMP, xylitol or erythritol, alone or in different associations, on microcosm biofilms, using pooled human saliva enriched or not with *S. mutans* as a pathogen. The null hypothesis of the study was that the antibiofilm effect of the actives alone would not be significantly different from those observed for the actives in different associations.

Materials and methods

Saliva collection

This study was approved by the Medical Ethical Committee of the VU University Medical Center 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 two hours before salivary donation. All saliva obtained was 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 (Exterkate et al., 2010).

Saliva-derived biofilm formation and exposure to the actives

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., (2010). In brief, 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 aluminum

foil and autoclaved at 121 ºC. The lid was fitted on top of flat-bottomed 24-well plates (Greiner Bio-One, Alphen a/d. Rijn, The Netherlands) (Fig. 1).

The cover slips were 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), 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 $CaCl_2$, 1 mg hemin (Sigma H1652), 0.2 mg vitamin K1, and 50 mmol/l PIPES, pH 7.0 (McBain et al., 2005).

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% N_2, 10% CO_2, 10% H_2) for 8 h in order 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 actives by adding them to the growth medium (McBain). The solutions were prepared by weighing and diluting the following actives: Xylitol (Sigma-Aldrich – St. Louis, MO, USA), Erythritol (Cargill, Vilvoorde, Belgium), Trisodium trimetaphosphate (Sigma-Aldrich Sigma-Aldrich – St. Louis, MO, USA), or Sodium fluoride (Merck – Darmstadt, Germany), alone or in different associations, in order to achieve final concentrations of 4.8%, 1.2%, 0.075%, and 0,013% (60 ppm F), respectively. The experimental groups in this study were: Pure McBain (negative control = “NC”), NaF 0.07% (positive control – 330 ppm F – “330F”), Xylitol 4.8% (“X”), Erythritol 1.2% (“E”), Sodium trimetaphosphate 0.075% (“TMP”), NaF 60 ppm F (“60F”), “X+E”, “60F+TMP”, “TMP+X+E”, “60F+X+E”, and experimental solution containing the four actives (60F+TMP+X+E – “EXP”). All compounds were filter-sterilized to obtain an aseptic condition. The concentrations were adopted to achieve 30% of the initial concentrations used in a previous study, in which the actives were administered as toothpastes (Marcato et al., 2021). To certify the presence of *S. mutans* in the biofilms, thus ensuring highly cariogenic conditions, this bacterium was added to the biofilm in a different set of experiments. For this, a *S. mutans* strain (C180-2) was pre-cultured in a BHI broth medium (BHI Agar; Difco, Le Pont de Claix, France) for 24 h. Subsequently (on the second day of biofilm formation), a 1:50 overnight pre-culture was added to the biofilm (Sampaio et al., 2022).

Twice daily (at 8 a.m. and 16 p.m.), the medium was replenished with fresh McBain medium containing the actives according to the experimental groups. In addition, the biofilms were grown in an unbuffered McBain medium supplemented with 0.2% sucrose (v/v) during the day-period, and in a McBain medium containing 50 mmol PIPES as a buffer, without the addition of sucrose in the nigh-period, from the second day onwards (Exterkate et al., 2010). 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., (2000). In summary, at the end of the biofilm formation period (96 h after 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% N_2, 10% CO_2, 10% H_2) for 3 h at 37 ºC to allow lactic acid production. Then, BPW solutions were analyzed for lactic acid concentrations using a colorimetric assay. Lactate standards were used for calibration.

Colony-forming unit 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 removed 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% N_2, 10% CO_2, 10% H_2) for 72 h at 37 °C (Exterkate et al., 2010). Bacterial morphology was considered for *S. mutans* counts, given the unselective nature of SAB.

Evaluation of the buffering capacity of the experimental solutions

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. For this, was used a pH electrode, previously calibrated with pH 4.0 and 7.0.

Statistical analysis

All experiments were performed in biological triplicate, on three separate occasions (n = 9). Statistical analyses were performed at a 5% significance level using the SigmaPlot software (version 12.0; Systat Software Inc., San Jose, USA). For the total CFU-counting and production of lactic acid, data (log_{10}-transformed) passed normality test (Shapiro-Wilk's test) and were submitted to one-way ANOVA, followed by Student-Newman-Keuls' post hoc test. *S. mutans* CFU-counting data (log_{10}-transformed) did not present normal distribution, so they were analyzed by Kruskal-Walli’s test, followed by Student-Newman-Keuls' *post hoc* test. Data on spent medium's pH presented normal distribution (Shapiro-Wilk's test) and were submitted to two-way, repeated measures ANOVA, followed by Student-Newman-Keuls' test.

Results

All treatment groups had significantly lower total CFU values compared to CN, except for the TMP group alone. In general, the greatest reduction was observed for 330F, followed by 60F+TMP, EXP and 60F groups. A synergistic effect between 60F and TMP was observed for saliva-derived microcosm biofilms, both supplemented or not with *S. mutans*. The groups containing polyols alone or in association also showed reductions, however, they were more subtle (Fig. 2a, and 2b).

A similar pattern was observed for CFU counts of *S. mutans* from saliva-derived microcosm biofilms supplemented with this bacterium, in which the 330F and 60F+TMP groups showed the greatest reductions (~3 $\log_{10}$) compared to the CN group (Fig. 3). On the other hand, for the biofilms not supplemented with *S. mutans*, the presence of the bacteria was not detected.

For lactic acid production, treatments with 330F or 60F+TMP promoted the greatest reductions in lactic acid production compared to the other groups in saliva-derived microcosm biofilms supplemented or not with *S. mutans*. The presence of the polyols did not promote any reductions in lactic acid production, as the values obtained were not significantly different from those of the CN (Fig. 4a and 4b).

As for the pH in the spent medium of the "day-period" (e.g., containing sucrose unbuffered McBain medium) of saliva-derived microcosm biofilms supplemented or not with *S. mutans*, the CN group showed the lowest pH value, followed by the groups containing F and polyols, alone or in association. The groups that contained TMP, either alone or in different associations, presented the highest pH values (close to neutrality). Conversely, the pH values in the spent medium of the night-period (e.g., buffered McBain medium, without sucrose) showed no significant differences between the pH values, with all values close to neutral (Fig. 5a and 5b).

Discussion

Dental caries is a disease mediated by biofilms formed by a consortium of different microbes, and modulated by the diet in the presence of fermentable sugars (Machiulskiene et al., 2020). Furthermore, it is well-established that most of the microorganisms involved in this pathogenic process act as producers of organic acids that may lead to hard tissue demineralization (Featherstone, 2008). Thus, acid production may be regarded as an important parameter of the cariogenic potential and virulence of oral biofilms. This study showed that solutions containing F, TMP, X or E, alone or in different associations, affected the viability and metabolism of total biofilms and *S. mutans*, thus leading to partial rejection of the null hypothesis.

Overall, the data show a pattern of dose-response reduction promoted by F on the counts of colony-forming units (Fig. 3). These effects were somehow

expected since this ion has the ability to inhibit the metabolism of microorganisms related to dental caries (Buzalaf et al., 2011). As for TMP alone, no reducing effect was observed, agreeing with data in the literature showing that this phosphate does not present an effect on the microbiological composition of biofilms, but rather reductions in metabolic activity, total biomass, and extracellular matrix components (Cavazana et al., 2019; Finn et al., 1978). Interestingly, when F was associated with TMP, these compounds showed a synergistic and potentiating effect, promoting significant reductions in biofilm growth, expressly by reducing total load and *S. mutans* counts which, in turn, were reflected in lactic acid production. These findings are in agreement with a previous study of *S. mutans* and *C. albicans* biofilms, showing that F+TMP significantly reduced *S. mutans* loads (Cavazana et al., 2019). Such effects may be related to the ability of TMP to bind to metallic ions (i.e., Ca^{2+} and $Mg^{2+)}$ present in the cell wall of Gram-positive bacteria, thus increasing cell permeability, causing a reduction in metabolism and leading to a reduction in the microbiological composition of the biofilm (Flemming & Wingender, 2010; Lee, Hartman, Stahr, Olson, & Williams, 1994).

S. mutans has the ability to produce high amounts of extracellular matrix, transport and metabolize different carbohydrates, converting them into lactic acid as an end product, thus promoting a highly acidic microenvironment and leading to hard tissue demineralization (Kilian et al., 2016; Klein, Hwang, Santos, Campanella, & Koo, 2015). This pathogen has been studied and demonstrated important virulent factors that are related to dental caries (Klein, Hwang, Santos, Campanella, & Koo, 2015). For this reason, this microbe was incorporated into the salivary inoculum to mimic highly cariogenic conditions and ensuring its presence in the studied biofilm (Sampaio et al., 2022).

As for the polyols, either alone or in association, they promoted slight effects in reducing the number of cells for total load and *S. mutans* when compared to the positive control (Fig. 3), what is in line with previous studies using single- and dual-species biofilms (Eskandarian et al., 2017; Giertsen, Arthur, & Guggenheim, 2011). Most studies suggest that polyols affect *S. mutans* cells by directly acting to reduce acidogenicity and inhibit their growth (Tanzer, Thompson, Wen, & Burne, 2006; Trahan, Bareil, Gauthier, & Vadeboncoeur, 1985). This action is due to the inability of *S. mutans* to metabolize these polyols, and consequently, the whole process of

maintaining this molecule inside the microorganism causes an energy expenditure, which is consumed and not replaced, thus leading to a reduction in the number of microbial loads (Hashino et al., 2013; Mäkinen, 2000; Park et al., 2014; E. M. Söderling & Hietala-Lenkkeri, 2010; Tanzer et al., 2006). In addition, for the experimental group (containing all actives), the effects of the polyols were potentiated by the other actives regarding total and *S. mutans* loads of biofilms. This potentiating effect can be explained by the previously mentioned effects promoted by F and TMP, coupled with the effect of polyols recently discussed. These interactions lead to changes in cellular metabolism, thus promoting the results expressed here.

The results found for the reduction in lactic acid production followed the same pattern as those for the total count and *S. mutans* microbial load, except for polyols alone or when associated with TMP (Fig. 4). As mentioned above, F and TMP act on bacterial metabolism. This effect was observed again in this variable, where these active agents in association promoted statistically significant reductions compared to the other groups. Despite these interesting data, the groups containing the polyols did not show any pattern of reduction in lactic acid formation. This lack of effect can be explained by the complexity of the model used, and the low concentrations of the active agents used, since most studies use static models of biofilms formed on polystyrene plates using single- or dual-bacterial species and high concentrations of the active compounds (Brambilla, Ionescu, Cazzaniga, Ottobelli, & Samaranayake, 2016; Cannon et al., 2020; Chan et al., 2020). Furthermore, data in the literature suggests that when the simultaneous presence of xylitol and sucrose in the medium occurs, sucrose appears to repress the inhibitory effect of xylitol on *S. mutans* cells (Ainamo, Asikainen, & Ainamo, 1978; Chan et al., 2020). This may be attributed to the fact that these microorganisms prefer the metabolization of glucose and fructose compared to xylitol (Chan et al., 2020). However, clinically, it is unlikely that less of this type of carbohydrate occurs in the oral cavity since much of the diet consists of fermentable carbohydrates. Another factor may be the age of the biofilms evaluated. Previous studies have shown that these polyols in biofilms (64.5 h) did not show inhibitory effects on *S. mutans* cells (Giertsen et al., 2011). Moreover, the best effects of reducing the formation of microbial inhibition were presented until the first 24 h of biofilm formation, suggesting that the polyols have better action in young biofilms (Kościelniak et al., 2019; Loimaranta et al., 2020).

Additionally, the pH values from the spent medium during the day- and night-period were analyzed. The literature shows that TMP exhibits buffering capacity due to its chelating characteristic (Cavazana et al., 2020; Klompmaker, Kohl, Fasel, & Mayer, 2017). In this experiment, when analyzed the pH values from the spent medium during the day-period (without buffering and supplemented with 0.2% sucrose), the groups that had TMP in the association were close to neutral values, indicating and confirming the buffering capacity of this phosphate (Fig. 5). The present study is consistent with the findings of Cavazana et al., (2020), where they evaluated the pH of mixed biofilms of groups treated with F + TMP before and after exposure to cariogenic challenges and observed that the pH promoted the highest values both before and after the challenges, keeping them closer to neutrality.

Some limitations of this study should be addressed, such as the use of low concentration of the actives due to the solubility of the polyols, in which high concentrations could not be feasible due to the difficulty in dissolving the crystals of the polyols altering their effects. Thus, this work became a proof-of-principle study in which low concentrations were tested in order to verify the viability of the actives in this model. Another point is the continuous presence of the actives in the microcosm biofilm model, which does not occur under clinical conditions, since these actives have their greatest contact time with the microenvironment and oral cavity during oral hygiene. Despite these limitations, this study promotes the use of new active options against oral microorganisms related to dental caries and contributes to the growth of the body of evidence on this subject in the literature. Future studies involving complementary analysis methods and different abiotic substrates, such as hydroxyapatite disks, should be considered to understand the exact mechanisms of these actives on polymicrobial biofilms.

Conclusion

Based on our results, we can conclude that solutions of F, TMP, xylitol/or erythritol alone or in combination showed significant reductions in growth and lactic acid production in oral microcosm biofilms and maintained the pH of the medium close to neutral.

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Figures

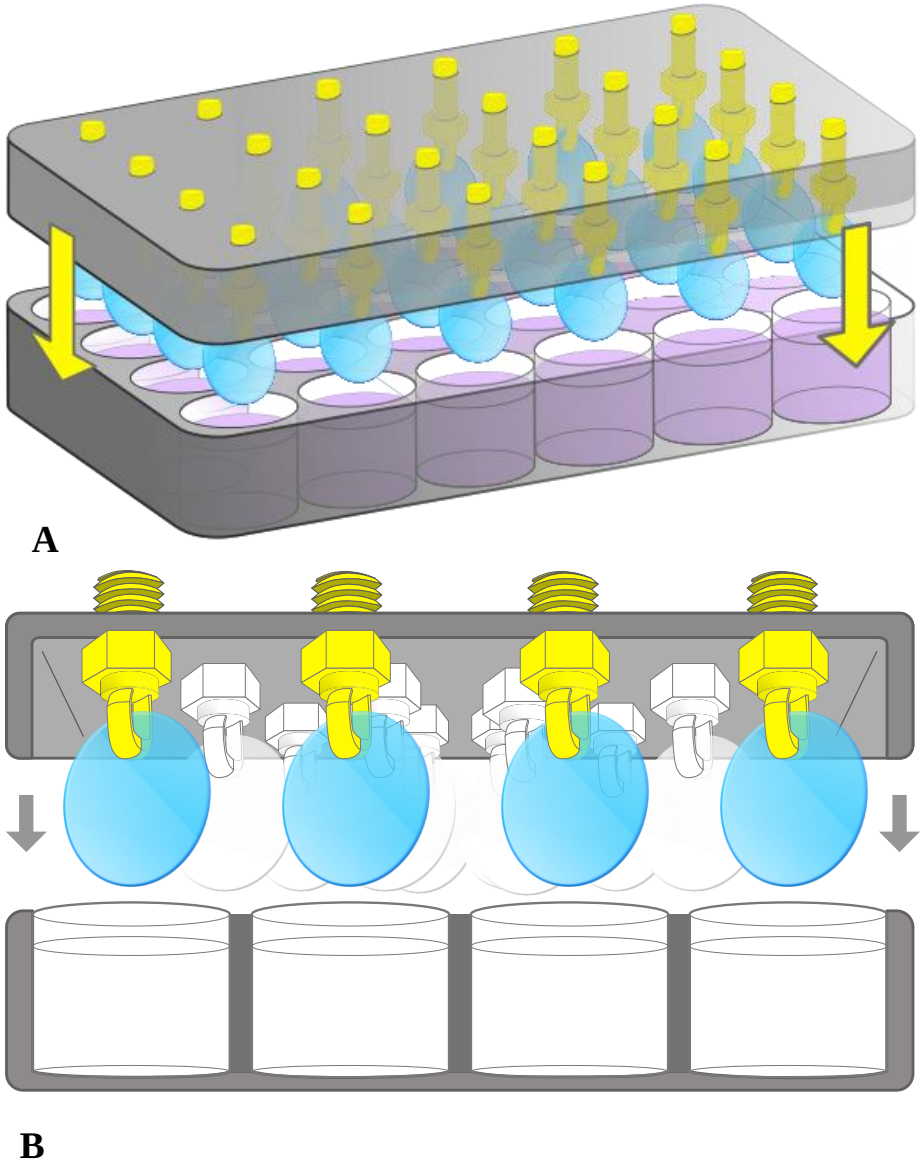

Fig. 1. A: AAA-model with glass cover lips attached to a stainless-steel lid using nylon clamps. B: The lid is fitted on top of flat-bottomed 24-well plates.

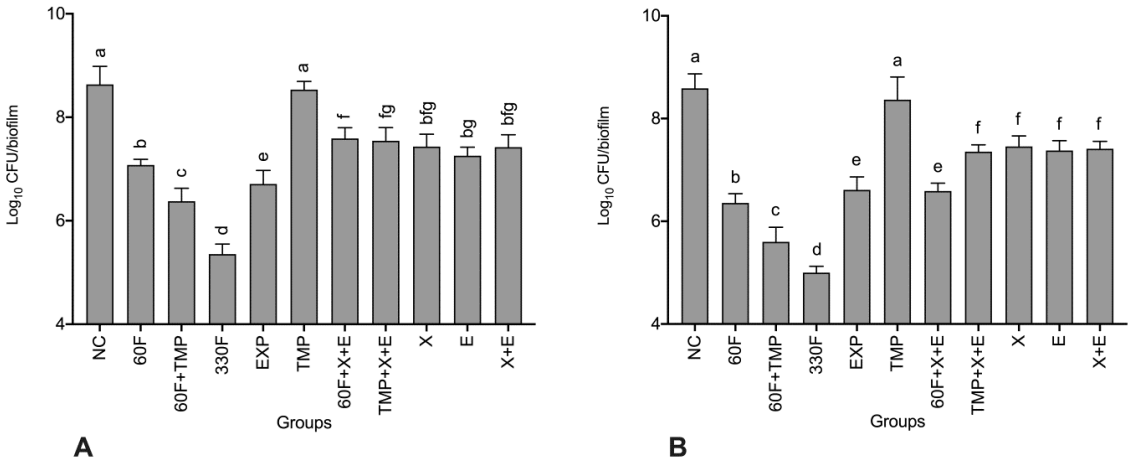

Fig. 2. Logarithm of total colony-forming units for (A): saliva-derived microcosm biofilms and (B): saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2) (ANOVA and Student-Newman-Keuls' tests, $p<0.05$, n=9). Error bars denote the SD of the means. Different uppercase letters symbolize statistical differences among the groups. NC: negative control (untreated biofilms), “60F” = 60 ppm F; “330F” = 330 ppm F; “TMP” = 0.075% Sodium trimetaphosphate; “X” = 4.8% Xylitol; “E” = 1.2% Erythritol.

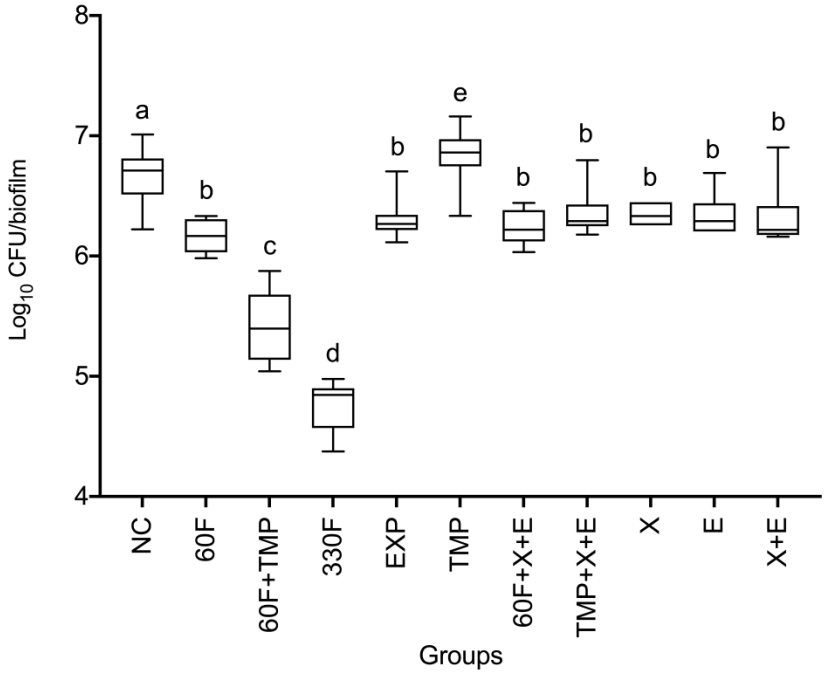

Fig. 3. Logarithm of colony-forming units of *Streptococcus mutans* from saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2). (Kruskal-Wallis and Student-Newman-Keuls' tests, $p<0.05$, $n=9$). Bars denote the interquartile ranges. Different uppercase letters symbolize statistical differences among the groups. NC: negative control (untreated biofilms), “60F” = 60 ppm F; “330F” = 330 ppm F; “TMP” = 0.075% Sodium trimetaphosphate; “X” = 4.8% Xylitol; “E” = 1.2% Erythritol.

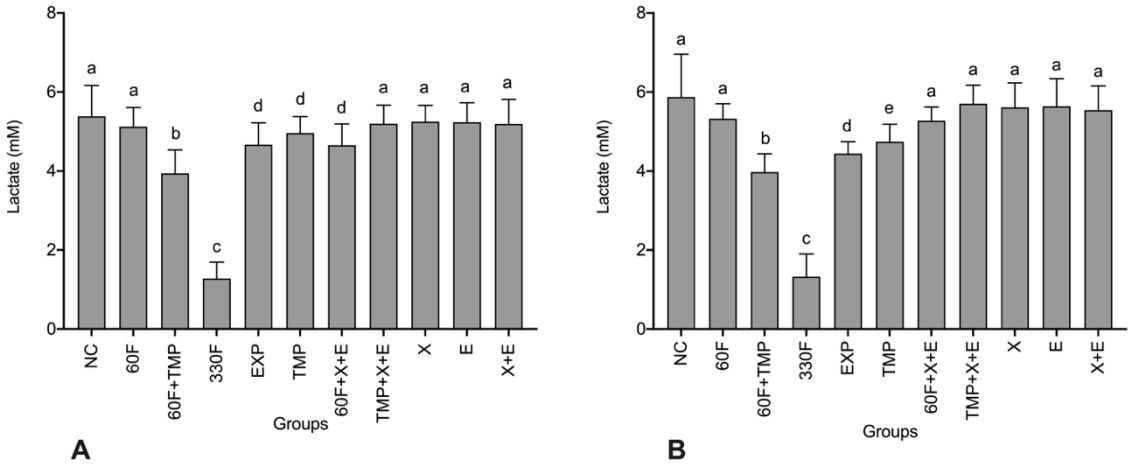

Fig. 4. Concentration of lactate (mM) produced by (A): saliva-derived microcosm biofilms and (B): saliva-derived microcosm biofilms supplemented with *Streptococcus mutans* (C180-2). (ANOVA and Student-Newman-Keuls' tests, $p<0.05$, n=9). Error bars denote the SD of the means. Different uppercase letters symbolize statistical differences among the groups. NC: negative control (untreated biofilms), "60F" = 60 ppm F; "330F" = 330 ppm F; "TMP" = 0.075% Sodium trimetaphosphate; "X" = 4.8% Xylitol; "E" = 1.2% Erythritol.

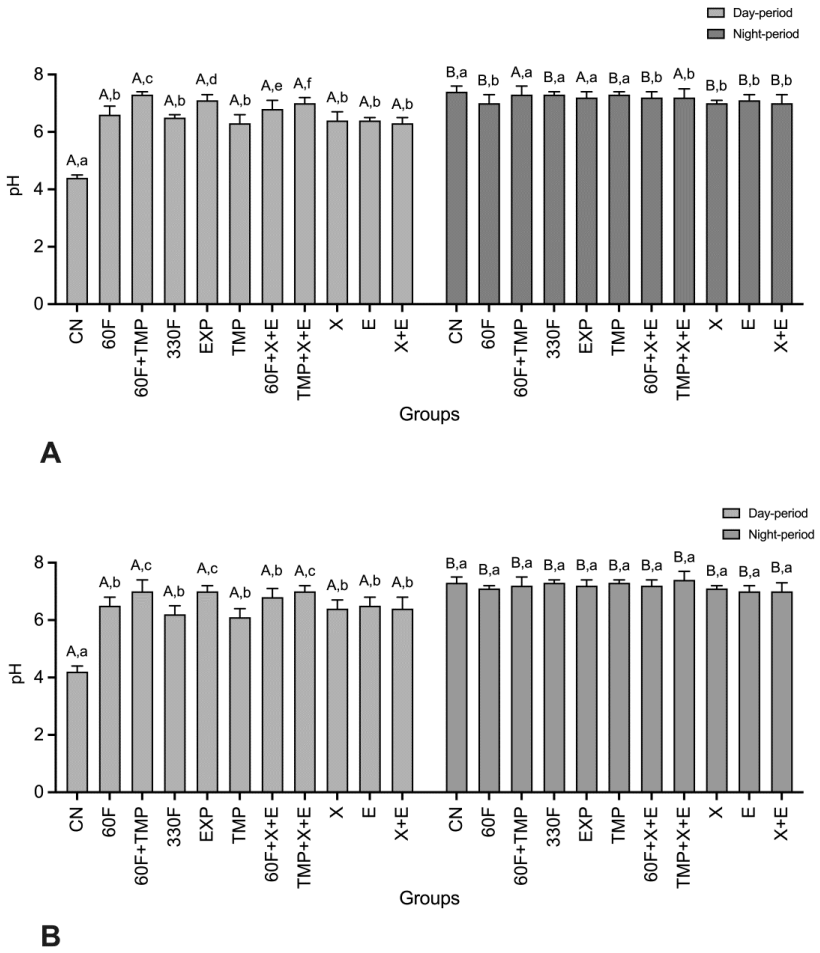

Fig. 5. Mean (SD) pH of the spent medium of saliva-derived microcosm biofilms from the day-period (unbuffered McBain medium supplemented with 0.2% sucrose) and night-period (buffered McBain medium without sucrose). Distinct upper-case letters indicate significant differences between day and night within each treatment solution. Different lower-case letters represent significant differences among the groups within a day and night periods of A: saliva-derived microcosm biofilms and B: saliva-derived microcosm biofilms supplemented with *S. mutans* (C180-2). 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). NC: negative

control (untreated biofilms), “60F” = 60 ppm F; “330F” = 330 ppm F; “TMP” = 0.075% Sodium trimetaphosphate; “X” = 4.8% Xylitol; “E” = 1.2% Erythritol.

ANEXOS

ANEXO A

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ANEXO B

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AUTHOR INFORMATION PACK

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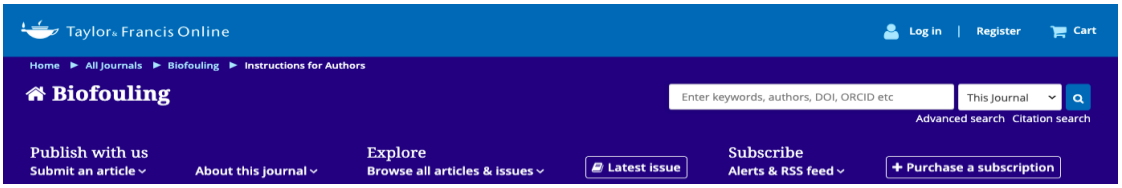

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ANEXO D

Normas Archives of Oral Biology

A Multidisciplinary Journal of Oral & Craniofacial Sciences

AUTHOR INFORMATION PACK

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Developmental biology Cell and molecular biology Molecular genetics Immunology Pathogenesis Microbiology Biology of dental caries and periodontal disease Forensic dentistry Neuroscience Salivary biology Mastication and swallowing Comparative anatomy Paeleodontology *Archives of Oral Biology* will also publish expert reviews and articles concerned with advancement in relevant methodologies.The journal will only consider clinical papers where they make a significant contribution to the understanding of a disease process.Journal Metrics

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GUIDE FOR AUTHORS

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Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter4).

Reference to a chapter in an edited book:

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