

RESEARCH ARTICLE

Biofilm extracellular polysaccharides degradation during starvation and enamel demineralization

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Abstract

This study was conducted to evaluate if extracellular polysaccharides (EPS) are used by *Streptococcus mutans* (*Sm*) biofilm during night starvation, contributing to enamel demineralization increasing occurred during daily sugar exposure. *Sm* biofilms were formed during 5 days on bovine enamel slabs of known surface hardness (SH). The biofilms were exposed to sucrose 10% or glucose + fructose 10.5% (carbohydrates that differ on EPS formation), 8x/day but were maintained in starvation during the night. Biofilm samples were harvested during two moments, on the end of the 4th day and in the morning of the 5th day, conditions of sugar abundance and starvation, respectively. The slabs were also collected to evaluate the percentage of surface hardness loss (%SHL). The biofilms were analyzed for EPS soluble and insoluble and intracellular polysaccharides (IPS), viable bacteria (CFU), biofilm architecture and biomass. pH, calcium and acid concentration were determined in the culture medium. The data were analyzed by two-way ANOVA followed by Tukey's test or Student's *t*-test. The effect of the factor carbohydrate treatment for polysaccharide analysis was significant ($p < 0.05$) but not the harvest moment ($p > 0.05$). Larger amounts of soluble and insoluble EPS and IPS were formed in the sucrose group when compared to glucose + fructose group ($p < 0.05$), but they were not metabolized during starvation time (S-EPS, $p = 0.93$; I-EPS, $p = 0.11$; and IPS = 0.96). Greater enamel %SHL was also found for the sucrose group ($p < 0.05$) but the demineralization did not increase during starvation ($p = 0.09$). In conclusion, the findings suggest that EPS metabolization by *S. mutans* during night starvation do not contribute to increase enamel demineralization occurred during the daily abundance of sugar.

Introduction

Dental caries is a biofilm-sugar related disease that depends on biofilm accumulation on tooth surface and its frequent exposure to dietary carbohydrates [1]. The cariogenic biofilm forms and grows on dental surfaces in a dynamic condition in which the exposure to dietary carbohydrates occurs intermittently [2]. During the day, the biofilm is frequently exposed to short periods of great amount of carbohydrates, followed by long periods of non-exposure between

the meals and overnight. These episodes are known as "feast" and "famine" periods and they are determinant for bacterial metabolism and biofilm growth [3, 4, 5]. In "feast" periods, bacterium such as *S. mutans* is able to store the excess of available carbohydrate as intracellular polysaccharides (IPS), which act as an energy reserve source in "famine" periods [6, 7]. Besides the IPS storage, glucosyl- and fructosyltransferases enzymes produced by *S. mutans* synthesize extracellular polysaccharides (EPS) when sucrose is the available carbohydrate [8, 9, 10].

EPS produced from sucrose contribute to microbial attachment and shifts on matrix tridimensional organization, mainly enhancing its porosity [11, 12, 13]. This structural change favors acid diffusion through the biofilm and pH fall next to the tooth surface, increasing enamel demineralization [14]. Additionally, it has been suggested that EPS could be an extracellular energy reserve, in which soluble glucans and fructans could be degraded by specific hydrolases, releasing glucose and fructose to be metabolized, producing acids [10, 15, 16, 17].

Once the degradation of EPS might occur during "famine" periods, the acid produced in a non-removed biofilm may extend the enamel demineralization especially during the night when salivary flow rate is low [18]. Furthermore, the effect of EPS metabolization on enamel demineralization has not been experimentally shown. The degradation of EPS has only been studied using planktonic cells [19, 20] and it was already reported that isolated or non-adsorbed enzymes could not simulate what happens in an organized biofilm [12, 21, 22]. Moreover, previous studies indicated that bacteria under starvation moments would have a different metabolic activity from those grown under constant nutrient exposure [23,24].

Therefore, considering that there is no data evaluating the effect of EPS degradation on enamel demineralization, the present study was carried out using a *S. mutans* cariogenic biofilm model subjected to intermittent periods of abundance and starvation of sucrose or equimolecular mixture of glucose and fructose. While sucrose is a source for IPS and EPS storage during energy abundance, glucose and fructose are only a source for IPS synthesis. Thus, it was possible to evaluate the degradation of EPS as an energy source during overnight starvation of carbohydrate and the consequent effect on enamel demineralization.

Materials and methods

Experimental design

An *in vitro*, randomized and blinded factorial (2x2) study was conducted using a validated cariogenic biofilm model [5, 25, 26]. The study was approved by the Research and Ethics Committee of the Piracicaba Dental School, University of Campinas (142/2014). The factors under study were type of carbohydrate (sucrose or a mixture of glucose + fructose) to feed the biofilms during the day, and biofilm harvest moment (at the end of the day after the last sugar treatment, situation of sugar abundance, or after overnight starvation), generating 4 experimental groups: (1) glucose + fructose abundance, (2) glucose + fructose starvation, (3) sucrose abundance, and (4) sucrose starvation. Sucrose and glucose + fructose are acidogenic carbohydrates and are stored as IPS, but only sucrose is substrate for EPS formation. *S. mutans* UA159 biofilms were formed on bovine enamel slabs, selected by surface hardness and randomized into the experimental groups (n = 12/group). The saliva-coated slabs were used to biofilms grown in ultrafiltered tryptone-yeast extract broth (UTYEB). The biofilms were exposed 8 times a day to 5.25% glucose + 5.25% fructose solution or 10% sucrose solution. Biofilm samples were harvested during two moments, at the end of the 4th day and on the morning of the 5th day, conditions of sugar abundance and starvation, respectively. Intracellular and extracellular polysaccharides (soluble and insoluble), biofilm dry weight and the number of viable cells were analyzed. During the biofilm growth, the culture medium was changed twice daily and the pH and calcium concentration were measured. The concentration of organic compounds

(acids and ethanol) present in the medium were also evaluated. Enamel demineralization was calculated by percentage of surface hardness loss (%SHL). Confocal laser scanning microscopy was used to visualize the biofilm organization (bacterial cells and EPS), the images were three-dimensional reconstructed and the biomass (cells and EPS quantified together) was calculated. The hypothesis under study was that EPS are metabolized during night starvation, increasing enamel demineralization occurred during daily sucrose exposure. Three independent experiments were carried out and the data were statistically analyzed according to the factorial design of this study, considering enamel slab as a statistical block ($n = 12$).

Enamel slabs preparation

Enamel slabs (4 x 7 x 1 mm) were obtained from bovine incisors [5]. The teeth were obtained from a local slaughterhouse, which is regulated by national legislation to follow sanitary standards. The bovine incisors were not collected for research purpose, and they were obtained after slaughtering. The teeth crowns were sectioned using a low-speed diamond blade to obtain enamel slabs. The surfaces were ground and polished using aluminum oxide abrasive papers (#400, #600 and #1200) and 1 μm diamond paste in a grinder machine (Phoenix Beta, Buehler, USA). The surface hardness (SH) was determined using a Knoop indenter (Future-Tech FM, Kawasaki, Japan) in which three indentations spaced 100 μm from each other were performed with 50 g load for 5 s on the polished surface center. The slabs with intra-variability higher than 10% were excluded and the selected slabs were randomized into the groups. The slabs were placed in 24-well culture plates, in vertical position using a metallic holder, and submitted to sterilization by exposure to ethylene oxide.

Biofilm model

The cariogenic biofilm model was previously described by Ccahuana-Vasquez and Cury (2010). *Streptococcus mutans* UA159 reference strain [27] was used in the experiment and cultures stored at -80°C were first grown on Columbia blood agar (CBA). To prepare the inoculum, *S. mutans* colonies were transferred to ultrafiltered tryptone-yeast extract broth (UTYEB) supplemented with 1% glucose and incubated overnight at 37°C , 10% CO_2 . The cells were centrifuged, washed with saline solution, and resuspended in fresh UTYEB. The cell suspension was standardized in a spectrophotometer at OD_{600} of 1.6 ± 0.5 to obtain the bacterial inoculum. Prior to bacterial cell adhesion, the slabs were immersed in human saliva to form the acquired pellicle. Fresh stimulated whole saliva was collected from the same two healthy donors for each experiment. Salivary flow was stimulated by chewing a plastic paraffin film and the saliva was collected in 50 mL polypropylene tubes on ice for 30 minutes. The saliva was pooled, centrifuged (10,000 g, 4°C , 10 min) and filtered (0.22 μm) [25]. The enamel slabs were immersed into human saliva at 37°C for 30 min [5] to form the acquired pellicle. Saliva-coated slabs were transferred to wells containing 2 mL of fresh UTYEB with 1% glucose and the bacterial inoculum (1:500 v/v), and were incubated for 8 h to promote initial adhesion of the microorganisms. Only in the adhesion phase, the UTYEB pH 7.0 was strongly buffered (10x higher than the usual phosphate concentration) to avoid pH drop and enamel demineralization during this phase. After adhesion, the slabs were transferred to fresh UTYEB pH 7.0 with 0.1 mM glucose (salivary basal concentration) and incubated overnight at 37°C , 10% CO_2 for 16 h. At the beginning of 2nd day, the biofilms were exposed to episodes of “feast” and “famine” comprised of 8 daily exposures to carbohydrate solutions: 10% sucrose or a combination of 5.25% glucose and 5.25% fructose at predetermined times (08:00, 09:30, 11:00, 12:00, 13:30, 15:00, 16:00 and 17:30 h) for 3 min [25, 26]. After the cariogenic challenge, enamel slabs were rinsed 3 times in 0.9% NaCl solution, and placed back into the culture media. The culture

media was changed twice daily, before the first challenge and after the last one. The pH was immediately evaluated after the change and the medium of each well was stored individually in microcentrifuge tubes at -80°C for later quantification of organic compounds and calcium concentration. At the end of the 4th day of biofilm formation, just after the last treatment, the biofilms were rinsed and remained during 10 min in fresh saline solution as standardized in a pilot study. This step was to avoid carrying any carbohydrate of the medium that could interfere in polysaccharide analysis. Half of the slabs with biofilms were collected for abundance condition evaluation. The other half was placed in fresh culture medium without glucose basal concentration, and at beginning of the 5th day, the biofilms were collected in starvation condition, after 16 h of night fasting (overnight starvation). The biofilm harvest was performed by sonication at 7 watts for 30 s as described by Ccahuana-Vasquez and Cury (2010). The slabs were used to evaluate enamel demineralization and the suspension was used for biofilm analyses.

Biofilm analyses

An aliquot of 100 μL of the biofilm suspension was ten-fold serially diluted in saline solution until $1:10^7$. Two drops of 20 μL of each dilution were plated on Todd-Hewitt broth (THB) plus agar and incubated at 37°C , 10% CO_2 for 48 h and the counts of the colony forming unit (CFU) was performed with the aid of a stereoscopic microscope [5, 28, 29]. The extraction of extracellular polysaccharides, soluble (S-EPS) and insoluble (I-EPS), and intracellular polysaccharides (IPS) was performed as described by Aires et al. (2008) from an aliquot of 400 μL of the sonicated biofilm suspension [30]. The amount of total carbohydrates was quantified by the phenol sulfuric method [31] using glucose as standard. Another aliquot of 400 μL of the sonicated biofilm suspension was added in pre-weigh microcentrifuge tubes to perform the biofilm dry weight analysis. The dry weight was determined by the difference between the final and initial weight of the microcentrifuge tubes [5, 29].

Culture medium analysis

The pH of the culture medium was evaluated twice a day at each medium change as an indicator of biofilm acidogenicity. The pH measurement was performed using a pH microelectrode (Accu-met; Cole-Parmer, USA) coupled to a pHmeter (Procyon SA-720, Olímpia, Brazil) calibrated with pH standards of 4.0 and 7.0, performed directly inside the wells, just after the medium change. Then, the culture medium was stored for calcium concentration analysis and organic compounds quantification. The Arsenazo III colorimetric method [32] was used to assess calcium concentration in 10 μL of medium from each sample. The absorbance was read at a wavelength of 650 nm in 96-well plates using a Multiskan Spectrum (Thermo Scientific) microplate reader [25]. The results were expressed as calcium concentration (mM) in function of the time of biofilm formation, and as cumulative calcium (μg) released from enamel (the sum of total quantity of calcium released from enamel to the medium until the harvest moments of abundance and starvation). The concentration of organic compounds (mM) was also performed at the two harvest moments. High Performance Liquid Chromatography (Alliance 2795, Waters, USA) was used with a refractive index detector and an Aminex HP-87H column (Bio-Rad Laboratories, USA) [33]. Standard curves of lactic, acetic and formic acids, and ethanol were used to calculate the concentration of each organic compound in samples. Triplicates of two independent experiments were used ($n = 6$).

Enamel demineralization assessment

After biofilm removal, the slabs were used to evaluate the enamel demineralization. The SH was performed again by three indentations 100 μm apart from the initial SH measurement and

the percentage of surface hardness loss (% SHL) was calculated as follows: (baseline SH—SH after assay \times 100)/baseline SH [14].

Confocal laser scanning microscopy (CLSM)

To visualize the architecture of biofilms formed under exposure to glucose + fructose or sucrose at abundance and starvation moments, additional enamel slabs were used. The extracellular matrix was labeled during the biofilm development with 1 μ M Alexa Fluor 647—dextran conjugate (molecular weight, 10,000; Excitation 650 nm/ Emission 668 nm; Thermo Scientific, USA) added to the culture medium. At the end of development, the biofilms were fixed in 4% paraformaldehyde (PBS, pH 7.2) for 30 min. *S. mutans* cells were stained with 2.5 μ M SYTO-9 green fluorescent nucleic acid (Excitation 485 nm/ Emission 498 nm; Thermo Scientific, USA) under protection from light for 20 min [34, 35]. The stained biofilms were mounted on coverslips containing 10 μ L of Mowiol, a glycerol based mounting medium for fluorescent staining [36]. The stained biofilms were examined using a DMI 6000 CS inverted microscope coupled to TCS SP5 computer-operated confocal laser scanning system (Leica Microsystems CMS, Mannheim, Germany) using 40x oil immersion objective (numeric aperture 1.25). An Ar-ion laser tuned at 488 nm and a He-Ne laser at 633 nm were used for excitation. Two enamel slabs were used for each condition evaluated. Five randomly sites were selected in the central area of the biofilms, avoiding the edges. Series of images were obtained from the enamel surface to the biofilm top at 1 μ m distance each in the Z axis [37]. The images of the biofilms were used for three-dimensional reconstruction with the ImageJ software [38], and to calculate the biofilm biomass ($\mu\text{m}^3/\mu\text{m}^2$) using the Comstat 2 software [39, 40]. Although the images of bacteria and EPS were obtained from different channels in CLSM, the biomass was calculated considering cells and EPS as a single channel. Therefore, biomass represents bacterial cells and EPS together.

Statistical analysis

Data were analyzed by two-way ANOVA, considering the factors carbohydrate (glucose + fructose or sucrose) and biofilm harvest moment (abundance or starvation), followed by Tukey's HSD Test. Student's *t*-test was used to evaluate differences between the carbohydrates for pH and calcium concentration values at each time point (8, 24, 32, 48, 56, 72, 80 and 96 h). The statistical analysis was done using SAS software (SAS Institute Inc., version 8.01, Cary, N.C., USA) employing a significance level fixed at 5%. Assumptions of homogeneity of variances and normal distribution of errors were checked for all tested response variables using the Kolmogorov-Smirnov test. Data that violated the assumptions were transformed to square-root (biofilm dry weight, %SHL and cumulative calcium released from enamel) and to \log_{10} (CFU counts, CFU counts/mg biofilm dry weight, polysaccharides, organic compounds).

Results

For the amounts of polysaccharides, the statistical analyses showed a significant difference for the factor carbohydrate ($p < 0.05$), however no differences were observed for the harvest moment (S-EPS, $p = 0.93$; I-EPS, $p = 0.11$; and IPS = 0.96) and for the interaction effect between the two factors (carbohydrate and harvest moment) (S-EPS, $p = 0.82$; I-EPS, $p = 0.08$; and IPS = 0.70). The same statistical pattern was observed for the variables: biofilm dry weight, CFU per biofilm dry weight, %SHL and biomass. The amounts of intracellular (IPS), and soluble (S-EPS) and insoluble (I-EPS) extracellular polysaccharides in biofilms formed under daily exposure to sucrose were higher, when compared to those found in biofilms exposed to glucose + fructose (Fig 1) ($p < 0.05$).

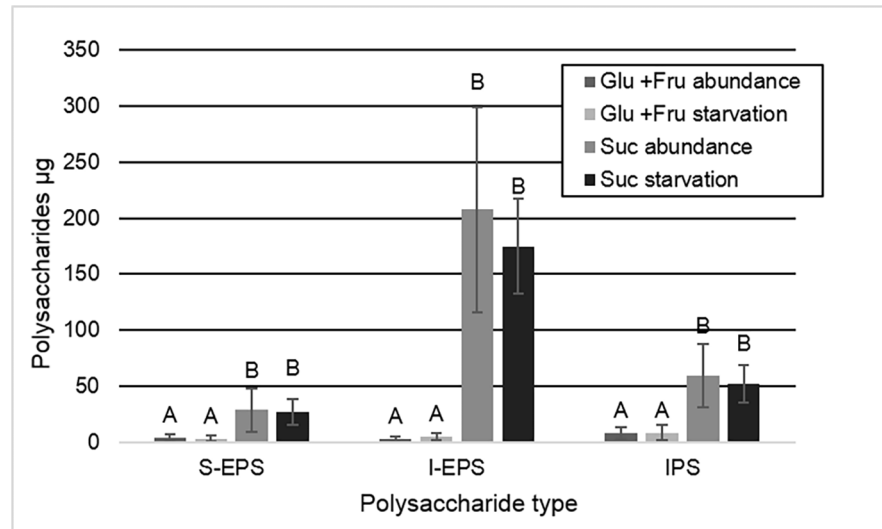


Fig 1. Amounts (µg) per enamel slab of extracellular polysaccharides, soluble (S-EPS) and insoluble (I-EPS), and intracellular polysaccharides (IPS) according to the treatments (glucose + fructose or sucrose) and the harvest moment (abundance or starvation). Distinct capital letters indicate significant statistically differences among groups for each polysaccharide type ($p < 0.05$) (Mean \pm SD; $n = 12$).

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The biofilm dry weight was much higher when the biofilm was formed under sucrose exposure than those formed with glucose + fructose ($p < 0.05$), and no significant change was observed after overnight starvation. On the other hand, there was no effect for the carbohydrate ($p = 0.20$) and neither for the harvest moment ($p = 0.07$) on the CFU counts (Table 1). When CFU counts were normalized by the biofilm dry weight, a greater number of viable cells per volume of biofilm was observed in the group exposed to glucose and fructose ($p < 0.05$) (Table 1). CLSM images showed extracellular polysaccharides (red) only synthesized in biofilms exposed to sucrose (Fig 2). In glucose + fructose group, only *S. mutans* cells (green) were visualized. It was also possible to observe that biofilm under sucrose treatment presented higher biomass (Table 1 and Fig 2), which is in accordance to EPS analysis (Fig 1) and biofilm dry weight (Table 1).

Table 1. Mean \pm SD ($n = 12$) of biofilm dry weight (mg), CFU counts (Log_{10}), CFU counts/mg biofilm dry weight (CFU $\text{Log}_{10}/\text{mg}$), %SHL, cumulative calcium released from enamel (µg) and biomass (µm³/µm²) according to the treatments (glucose + fructose or sucrose) and the harvest moment (abundance or starvation).

Carbohydrate	Biofilm harvest moment	Analysis					
		Biofilm dry weight (mg) ^a	CFU counts (CFU Log_{10}) [*]	(CFU Log_{10})/mg biofilm dry weight [*]	%SHL ^a	Cumulative Ca ⁺⁺ released from enamel (µg) ^a	Biomass [§] (µm ³ /µm ²)
Glucose + Fructose	abundance	0.4 \pm 0.2 ^A	7.4 \pm 1.1 ^A	12.3 \pm 1.1 ^A	14.5 \pm 7.1 ^A	24.7 \pm 14.8 ^A	22.3 \pm 5.9 ^A
	starvation	0.6 \pm 0.4 ^A	7.8 \pm 1.2 ^A	15.4 \pm 7.5 ^A	11.9 \pm 4.6 ^A	17.1 \pm 10.7 ^A	23.7 \pm 7.9 ^A
Sucrose	abundance	1.6 \pm 0.3 ^B	7.6 \pm 1.0 ^A	4.8 \pm .3.0 ^B	41.4 \pm 6.5 ^B	85.1 \pm 29.9 ^B	36.2 \pm 8.4 ^B
	starvation	1.6 \pm 0.3 ^B	8.3 \pm 0.5 ^A	5.4 \pm 1.2 ^B	34.7 \pm 7.7 ^B	33.2 \pm 24.4 ^A	36.1 \pm 7.1 ^B

Distinct capital letters indicate significant statistically differences ($p < 0.05$) among groups for each variable (values within columns).

^{*} The values were transformed to log_{10} .

^a The values were transformed to square-root.

[§] Biomass refers to bacterial cells and EPS

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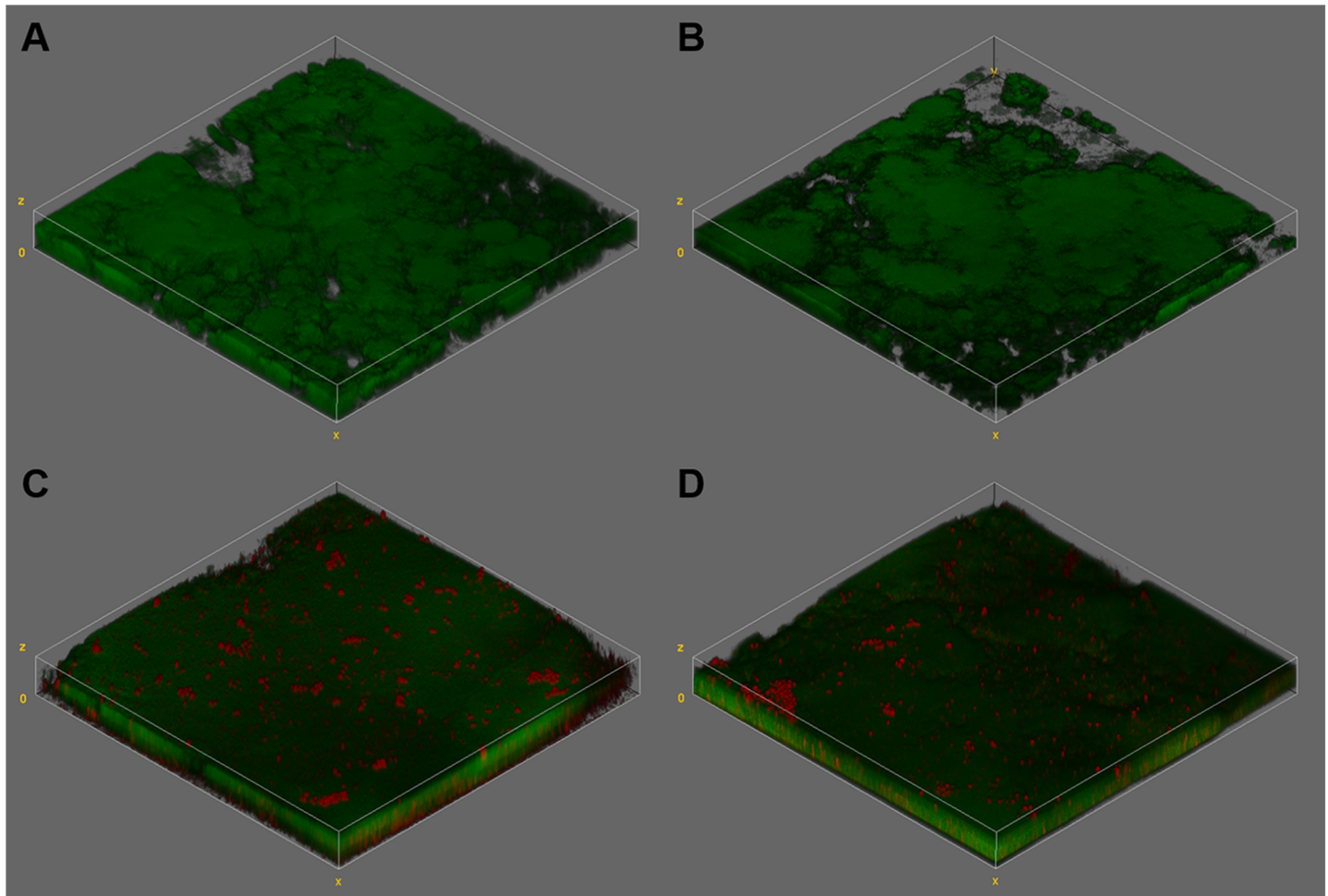


Fig 2. Tridimensional reconstruction of CLSM images of *S. mutans* biofilms formed under exposure to glucose + fructose (A and B) or sucrose (C and D) 8x/daily. Images A and C show biofilms visualized at abundance moment, while images B and D at starvation moment. In green, *S. mutans* cells stained with SYTO 9. In red, extracellular polysaccharides labeled with Alexa Fluor 647—dextran conjugate. Oil immersion objective of 40x (numeric aperture 1.25).

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The pH values of the culture medium were lower in the group exposed to sucrose. This difference was observed from 56 h, which corresponds to 2nd day of biofilm development under cariogenic challenge, when it started to be mature [36] (Fig 3). Lower amount of acids was produced by *S. mutans* when biofilm was formed under glucose + fructose exposure, when compared to sucrose ($p < 0.05$). Among the acids, lactic acid was the most produced in both treatment groups, showing significant effect for the factors carbohydrate and harvest moment ($p < 0.05$). The values of lactic acid produced by biofilms exposed to sucrose were 2 times higher than those observed in the group treated with the combined monosaccharides ($p < 0.05$). Acetic acid was produced in small amounts and only the carbohydrate factor had a significant effect ($p < 0.05$). Ethanol was also produced as a final compound of the carbohydrate metabolism being significant only to the harvest moment factor ($p < 0.05$) (Fig 4). In starvation condition, acid production was lower for both glucose + fructose and sucrose groups, but it occurred even after removal of all carbohydrate sources ($p < 0.05$).

The calcium concentration in culture medium during biofilm development was higher in sucrose group ($p < 0.05$). This higher quantity released from enamel was seen from 32 h of biofilm development and became more evident with the biofilm maturation (Fig 5), mainly at

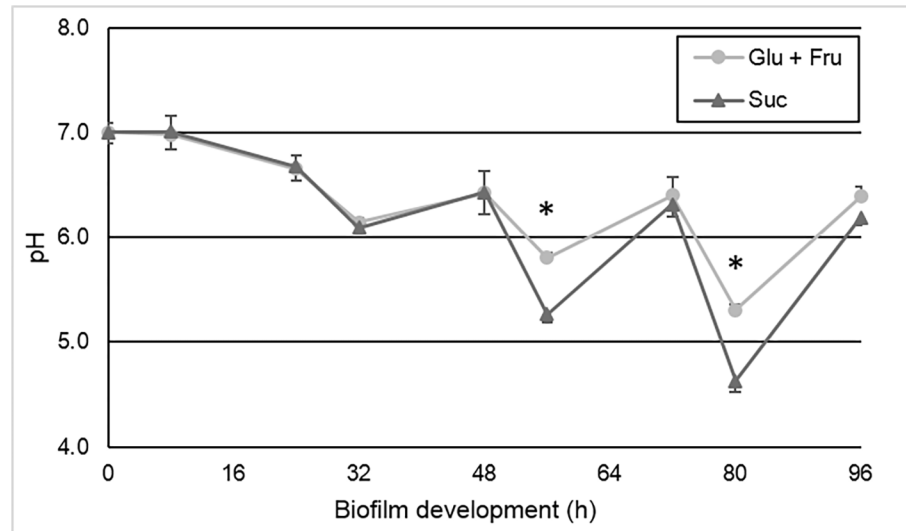


Fig 3. pH values of the culture medium according to the biofilm treatments (glucose + fructose or sucrose) and biofilm development time (h) as an indicator of biofilm acidogenicity. Time points at 80 h and 96 h refer to the harvest moment at abundance and starvation of carbohydrates, respectively. Asterisks indicate statistically significant difference between treatments at the time point evaluated ($p < 0.05$). (Means \pm SD; $n = 12$).

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the end of the 4th day (abundance condition) ($p < 0.05$). This behavior was consistent with the pH (Fig 3) and %SHL (Table 1) data. Higher %SHL was observed in enamel slabs in the sucrose group, which differed statistically from the group treated with the combined monosaccharides ($p < 0.05$). Under sucrose exposure, the enamel slabs lost around 30 to 40% of

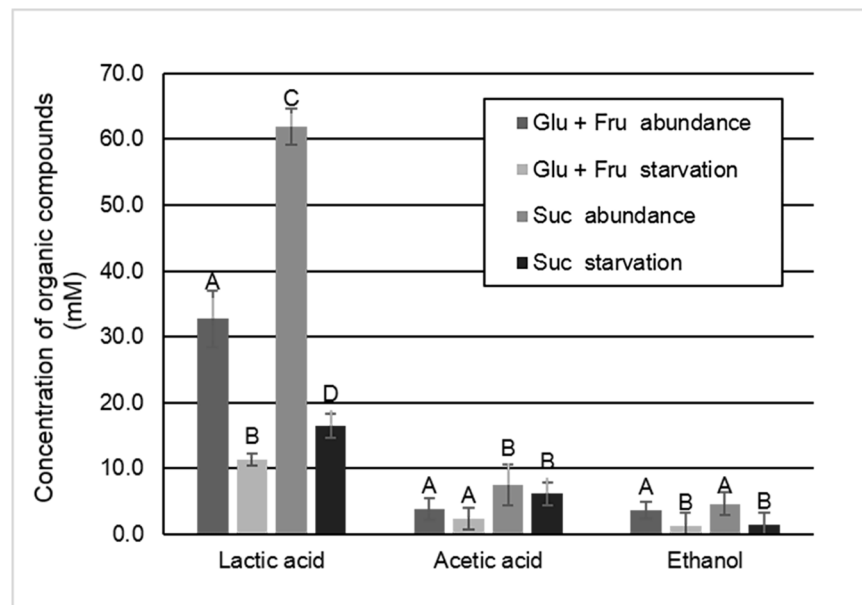


Fig 4. Concentration of organic compounds (mM) in the culture medium according to the biofilm treatments (glucose + fructose or sucrose) and the harvest moment (abundance or starvation). Distinct capital letters indicate significant statistical differences among groups for each type organic compound ($p < 0.05$). (Mean \pm SD; $n = 6$).

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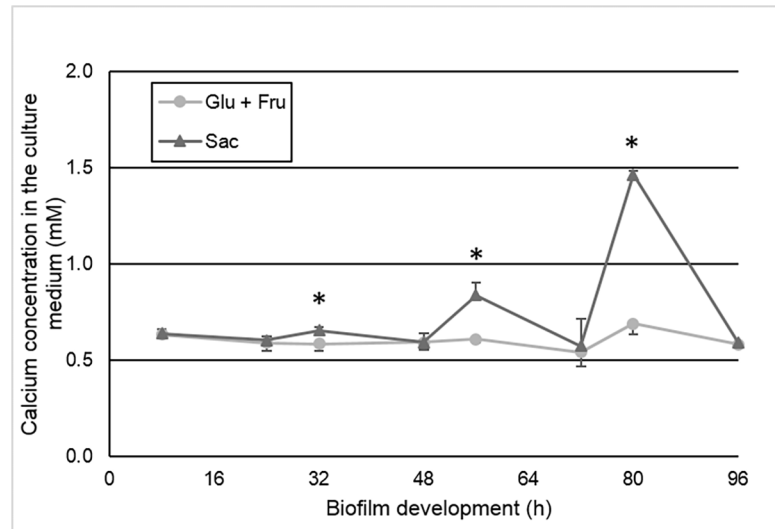


Fig 5. Calcium concentration (mM) in the culture medium according to the biofilm treatments (glucose + fructose or sucrose) and biofilm development time (h). Time points at 80 h and 96 h refer to the harvest moment at abundance and starvation of carbohydrates, respectively. Asterisks indicate statistically significant difference between treatments at the time point evaluated ($p < 0.05$). (Mean \pm SD, $n = 12$).

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hardness, while those exposed to glucose + fructose lost around 15%. No significant effect was observed in relation to harvest moment to the %SHL data (Table 1).

The cumulative calcium released from enamel showed higher amount of calcium released when the biofilm was exposed to sucrose, however only in the harvest moment of abundance ($p < 0.05$) (Table 1). The cumulative calcium values from the group exposed to sucrose at starvation period were similar to the groups exposed to glucose + fructose, regardless of the harvest moment (abundance or starvation).

Discussion

In this study, we used a validated *S. mutans* biofilm model for caries study [5] to evaluate the degradation of extracellular polysaccharides (EPS) as an energy source during overnight starvation of carbohydrate and the consequent effect on enamel demineralization. Once this approach has not been performed using biofilms, this study seems to be a pioneer in the field. The experimental design using different carbohydrates allowed us to develop biofilms with EPS (sucrose) or without them (glucose + fructose), however, for both groups, bacterial cells could also store intracellular polysaccharides (IPS), which could also be used as an energy source. Our findings showed no reduction in the amounts of EPS and IPS (Fig 1), and no increase in enamel demineralization after the overnight period of starvation (Table 1). The non-reduction of EPS and IPS quantities may indicate that they were not metabolized during the starvation period, or that they were metabolized in a small amount, just to maintain the bacterial basal metabolism, causing no additional damage to the enamel.

The lack of increase in enamel demineralization was confirmed not only by the percentage of surface hardness loss (%SHL) (Table 1), but also by the calcium concentration in the culture medium (Fig 5). Thus, the known cariogenicity of sucrose [8, 10, 41, 42] was evidenced in our study. The sucrose group presented %SHL almost 3 times higher than the glucose + fructose group ($p < 0.05$) (Table 1). However, no difference in the %SHL was observed between the abundance and starvation moments for each group. In order to verify the enamel

demineralization using a more sensitive analysis, we also evaluated the calcium concentration in the culture medium. Although higher amounts of calcium in the medium were observed for the sucrose group (Fig 5; $p < 0.05$) after the abundance period (80 h), no increase ($p > 0.05$) in the calcium concentration was detected when both groups were compared after the overnight starvation period (96 h). This finding could strengthen and confirm that there was no increase in enamel demineralization during the starvation period by the EPS or IPS use.

Calcium kinetics (Fig 5) inversely follows the pH's dynamic (Fig 3) occurred into biofilm [25]. Therefore, higher acid production caused higher enamel demineralization, releasing more calcium from the enamel to the culture medium. Lower pH values were observed for the group exposed to sucrose after the abundance period from the second day of cariogenic challenge (56 and 80 h) (Fig 3). Even using solutions with equimolar concentration of carbohydrates, the pH values for the sucrose group were lower than the glucose + fructose group. The lower pH is explained by the EPS in the matrix that are only synthesized in the presence of sucrose [8]. It is known that EPS change the biofilm structure by the increase of the matrix porosity, which favors acid diffusion and pH drops nearby the enamel surface [4, 11]. In addition, micro-compartments formed within the biofilm matrix could generate heterogeneous environments keeping the pH low for longer periods [13, 43], increasing the cariogenic challenge. In our study, during the cariogenic challenges, the acids produced by bacteria in the biofilm caused the enamel demineralization [44]. Calcium was continuously released from enamel to the biofilm, and from the biofilm to the medium. The same occurred for the acids produced. Therefore, the calcium and acid concentration in the medium reflect the cariogenic process occurred on the enamel surface in contact with the biofilm.

The higher acid production for the biofilm exposed to sucrose (Fig 4) cannot be explained either by the counts of *S. mutans* cells, because they were similar for the sucrose and glucose + fructose groups at both harvest moment (Table 1). The difference in the sucrose group was the presence of EPS in the matrix. In addition to modifying the biofilm architecture [43], the synthesized EPS also increased the biofilm volume (Table 1). The biofilm formed under sucrose exposure was 3 times heavier than the glucose + fructose group ($p < 0.05$) (Table 1), which could represent from 10 to 40% of the biofilm dry weight [10, 12]. Tridimensional images of the biofilms (Fig 2) and biomass data (Table 1) also showed that difference ($p < 0.05$). Taking into account CFU counts normalized by biofilm dry weight and biomass data, it was possible to infer that more cells per volume were present in the biofilm exposed to glucose + fructose, because in this condition there were no EPS. On the other hand, the biofilm exposed to sucrose showed less cells per volume, suggesting EPS could determine the bacterial density in biofilms [17]. The cells dispersed into EPS matrix would be easily exposed to sugars, due to higher porosity of the biofilm [11], which could allow more acid production and IPS storage, when compared to the packed cells in the biofilm without EPS (glucose + fructose). This could also explain the great amount of IPS formed in the group exposed to sucrose (Table 1). Thus, the increase in biofilm cariogenicity can be better understood by changes occurred in matrix composition and structure, and not simply by the counts of bacterial cells in biofilms [43].

The presence of EPS in dental biofilm matrix has been reported as an important virulence factor for caries development [8, 13, 14, 41, 43]. A study reported increased expression of a gene related to EPS degradation (*dexA*) in *S. mutans* mature biofilms [42], and another showed the non-utilization of IPS when sucrose was present [6], both suggesting that EPS from biofilm matrix could be used as an extracellular energy source [15, 17]. It was already demonstrated in planktonic cultures that other oral microorganisms could produce EPS and degrade them [20]. However, the EPS produced by *Streptococcus mutans* seem to be far less soluble and more resistant to dextranase action [20]. EPS degradation was not observed in our study using a *S. mutans* biofilm model. One of the reasons why there was no difference for EPS quantities

between the two harvest moments might be due to the starvation period. Maybe a longer fasting period could trigger the metabolization of polysaccharides by the cells to obtain energy. Another reason could be that bacterial cells just break and use small ending fragments of the EPS, which had not been detected by the phenol sulfuric method. Therefore, future studies using a longer period of starvation or a methodology using radiolabelled sucrose in the fructosyl (^3H) and glucosyl (^{14}C) moieties, instead of the phenol sulfuric method, might be useful to clarify EPS metabolization.

Considering that the used *S. mutans* biofilm model simulates the "feast" and "famine" episodes that occur into the mouth, the EPS metabolism during starvation may not be sufficient to increase the biofilm cariogenicity, but the high carbohydrate exposure frequency occurred during the day. In summary, the findings suggest that EPS metabolization by *S. mutans* during night starvation do not contribute to increase the enamel demineralization occurred during the daily abundance of sugar.

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