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Extended biofilm formation time by *Streptococcus sanguinis* modifies its non-cariogenic behavior, *in vitro*

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Abstract: Although the commensal *Streptococcus sanguinis [S. sanguinis]* is isolated from caries-free people, it can ferment carbohydrates producing acids. We aimed to characterize *S. sanguinis* cariogenic potential as a function of different enamel biofilm formation periods, *in vitro*. Saliva-coated enamel slabs were inoculated with *S. sanguinis* to form initial biofilms for 8, 12 or 16 h in presence of sucrose and followed by a period in medium with glucose for 16, 12 or 8 h, respectively, until completion of 24 h. To simulate cariogenic challenges, *S. sanguinis* biofilms were exposed to 10% sucrose for 5 minutes, 3x/day for 5 days. Biofilm biomass, viable cells, total proteins, intracellular and extracellular polysaccharides production, acidogenicity and enamel demineralization were determined. Biofilms of *Streptococcus mutans* [*S. mutans*] served as caries-positive control. Biofilms of *S. sanguinis* forming on enamel for 12 and 16 h showed higher demineralization than those formed during 8 h, but lower than *S. mutans* biofilms, regardless of the initial biofilm formation time. No differences were detected in the biofilm properties among the different biofilm formation times tested for *S. sanguinis*. Increased enamel initial biofilm formation time by *S. sanguinis* appears to induce a cariogenic potential, but lower than *S. mutans*.

Keywords: Dental Caries; Dysbiosis; Symbiosis; *Streptococcus sanguis*; *Streptococcus mutans*; Dental Enamel.

Introduction

Hundreds of bacterial species colonize the dental hard tissues, organized in highly complex communities referred to as dental biofilm.[1] In health, bacteria comprising the dental biofilm share multiple ecological micro-environments, surrounded by a polysaccharide matrix.[2,3] Under these conditions, most of the bacteria are considered commensals, that is, they live in an ecological niche without benefiting or harming the host.[4] Typically considered a commensal, *Streptococcus sanguinis* [*S. sanguinis*] has been abundantly isolated from dental biofilms of caries-free children[5] and adults.[6] Its ability to colonize the enamel has been studied, particularly in children.[7] Studies have shown that the relative presence of *S. sanguinis* in biofilms and saliva isolated from caries-free children and adults is higher than the number of cells from

caries-active individuals,[5,6] suggesting a health-protective role. A complete review on the commensal traits of *S. sanguinis* has been published.[8]

Under adverse environmental circumstances, for example upon frequent sugars exposure, an ecological imbalance or dysbiosis occurs, which induces important changes in the homeostasis of the health-associated dental microbiota.[3,9] Hence, bacteria that previously acted as commensal undergo a transition to pathobiont, becoming potentially pathogenic and capable of causing disease.[9] Thus, dental caries is currently better defined as a *"dysbiosis associated to pathobionts"* and not as an infectious-contagious disease as it was previously thought.[9,10] The dynamics of enamel colonization by the different bacterial species of the dental biofilm, and the timing in which it occurs, may predispose to the onset of the dysbiosis. Clinical trials show that *S. sanguinis* colonizes the oral cavity of toddlers during the eruption of the first teeth and that its colonization precedes that of *S. mutans*.[11] On the other hand, *in situ* studies have reported that during the first 4 to 8 h of biofilm formation, *Streptococcus spp.* are the pioneer colonizers of the enamel, particularly those of the *mitis* group.[12] Other studies indicate that the proportion of *Streptococcus spp.* increases from the first 12 h of adherence to dental enamel.[13] Clinically, refraining from toothbrushing for a period of time will increase colonization time by *Streptococcus spp*., which could become a key factor in the cariogenicity elicited by biofilms formed on enamel.[14] Yet, it is rather unclear whether variable initial colonization time by *S. sanguinis* may impact the final cariogenic traits of a mature biofilm. Therefore, the question is if allowing longer initial biofilm formation times and in the presence of an excess of sugars, a commensal and health-associated bacterium, such as *S. sanguinis*, might induce a cariogenic potential, for example, when longer periods of time without toothbrushing are allowed. Consequently, the aim of the present study was to compare enamel demineralization and the properties of *S. sanguinis* biofilms formed on enamel for different initial biofilm formation times, before being exposed to cariogenic challenges with sucrose. The results of this exploratory approach led us to rethink the role of this commensal bacterium in the ecology of the dental biofilm under an excess of sugars.

Methodology

Enamel slabs

Bovine incisors were obtained, disinfected with 5% sodium hypochlorite (NaOCl) and stored in distilled water up to 30 days before being used. Enamel slabs (4 mm x 7 mm x 1 mm) were prepared using a diamond saw blade (VC50 diamond Saw, LECO, St. Joseph, USA) and polished using an automatic polisher (SS200, LECO) followed by a sequential series of polishing discs (Soflex 1982F, 1982M and 1982C, 3M-Oral Care, St. Paul, USA). Initial surface microhardness $(SH)_i$ was determined by three Knoop indentations at 50 g for 5 s, performed 100 µm apart from each other, with a microhardness tester (402 MVD, Wolpert Wilson Instruments, Norwood, USA). Only enamel slabs with microhardness values of 364.19 ± 36.4 kg mm^{-2} (n = 48) were included in the study to allow similar initial conditions for all the slabs. Enamel slabs were sterilized by autoclaving at 121°C for 15 min. To simulate the acquired pellicle promoting bacterial adhesion on enamel,[15] slabs were conditioned for 30 min with human stimulated saliva. Saliva was sterilized by filtration through a 0.22 µm pore size filter (Rapid Flow, Nalgene, Rochester, USA). Fresh saliva was obtained from two healthy donors after 12 h of fasting. In order to prevent proteolysis, saliva was mixed with a 1/100 [v/v] solution of phenylmethylsulfonyl fluoride (PMSF; ThermoFisher Scientific, Waltham, USA), a protease inhibitor. Slabs were randomly arranged in wells of a 24-well culture plate (Costar®, Corning, New York, USA) containing 2 mL of saliva and suspended through metallic devices made from orthodontic wire.

S. mutans and *S. sanguinis* biofilms

Frozen stocks of *S. mutans* UA159 and of *S. sanguinis* SK36 were reactivated in brain heart infusion broth [BHI (contains glucose 2 g/L); Merck, Darmstadt, Germany] supplemented with 1% glucose and incubated at 37°C and 10% CO_2 (Incubator Panasonic, MCO-19M, Osaka, Japan) for 18 h. Slabs were inoculated with

100 µL of a *S. sanguinis* or *S. mutans* culture (A_{600nm} of 0.1, equivalent to 10^3 to 10^4 CFU/mL) in BHI medium containing 1% sucrose, previously prepared, to induce formation of an adhesive biofilm.[15] Three different initial colonization (biofilm formation) times were chosen, where inoculated bacteria (*S. sanguinis*) were allowed to adhere, form biofilms and mature for: 8, 12 and 16 h in the presence of 10% sucrose or 0.9% NaCl. A fourth group was inoculated with 100 µL of a culture of *S. mutans* in BHI medium containing 1% sucrose and the biofilms were allowed to mature for 8 h in the presence of 10% sucrose or 0.9% NaCl at 37°C and 10% CO_2.[16] To obtain mature biofilms before beginning the cyclic exposures to sucrose, enamel slabs/biofilms were allowed to grow in BHI medium supplemented with 0.1 mM glucose,[16] until completion of 24 h for all the groups. Thus, the slabs that had been inoculated with *S. sanguinis* to form biofilms for 8, 12 and 16 h, were matured for additional 16, 12 and 8 h, respectively.

Sucrose exposure to the biofilms

To study the demineralizing potential of the biofilms formed under the different initial biofilm formation times, they were challenged with sucrose according to a previously used *in vitro* caries model in our Laboratory.[16] Briefly, after obtaining a mature biofilm at 24 h, slabs were transferred to a new 24-well plate with BHI supplemented with 0.1 mM glucose. Each slab/biofilm was immersed in 10% sucrose solution[16] for 5 min, three times per day (8:30, 12:30 and 16:30 h). Immediately after the exposure, biofilms were washed with 0.9% NaCl and relocated in the original plate. Biofilms unexposed to sucrose and exposed to 0.9% NaCl three times per day for 5 min were used as negative controls. Spent culture medium was replaced twice per day, prior to the first (overnight) and after the last sucrose exposure. The trial extended for 5 days, time considered enough to produce demineralization on the slabs.[16] The full experiment was repeated independently, with each condition in triplicate (n = 6).

Biofilm acidogenicity

Slabs were exposed to 10% sucrose three times per day (feast) and then maintained with BHI supplemented with 0.1 mM glucose basal glucose conditions (famine-like situation), which led to a pH-cycling condition, mimicking the clinical situation.[17] Medium pH is a way to estimate acid production by bacterial biofilms, which in turn will demineralize hard dental tissues. Thus, to verify acid production by the biofilm, pH of the spent culture medium was measured after the daily cariogenic challenges and after the overnight period using a microelectrode (Orion 910500, Thermo Scientific, Waltham, USA) coupled with a pH meter (Orion Star A211, Thermo Scientific, Waltham, USA), during the entire length of the experiment. Measurements of the spent medium pH after a series of sucrose exposures were carried out at 32, 56, 80, 104 and 120 h.

Enamel demineralization

Demineralization was assessed through the Knoop's surface microhardness test [18]. Surface microhardness has been broadly used as a demineralization indicator [19] and it was validated for enamel caries.[16] After the five-day experimental period, biofilms were separated from the slabs by vortexing for 30 s at maximal speed (Maxi Mix II Type 37600 Mixer, Thermolyne, Dubuque, USA) in 0.9% NaCl. The resulting biofilm suspension was kept for further biofilm analysis. Slabs were mounted on a glass plate to obtain the final microhardness reading $(SH)_f$ (kg/mm^{-2}), through three indentations separated by 100 µm. Mean values of SH_i and SH_f measurements were used to calculate the percentage of SH loss (%SHL): (mean SH_i – mean SH_f) x 100/ mean SH_i.

Biofilm analysis

The previously obtained master biofilm suspension, detached from the slabs, was stored for further analysis of biomass, intra- and extracellular polysaccharides,[17] and total proteins[20] as it is summarized below:

a. *Biomass*: Sample dry weight was used to estimate biomass.[15] A 200 µL aliquot of the biofilm suspension was transferred to a previously weighed tube $(weight)_i$ and incubated with absolute ethanol at -20°C for 15 min. The resulting suspension was centrifuged for 10 min at 5,000 *g* and 4°C (Heraeus Megafuge

16R, ThermoFisher Scientific, USA), and the supernatant was washed with 500 µL of 75% ethanol and centrifuged under the same conditions mentioned above. Biofilms were desiccated through evaporation of all the liquid components in a multi-gas incubator at 37ºC for 24 h (Incubator MCO-19M Panasonic, Osaka, Japan) to obtain the final dry weight $(weight)_f$. The following formula was used to obtain the amount of biomass: $weight_f - weight_i$, expressed in mg/mL of biofilm suspension.

b. *Intracellular and extracellular polysaccharides*: A previously described method was used for the analysis of polysaccharides.[21] Three different fractions of polysaccharides were obtained: soluble extracellular polysaccharides (SEPs), insoluble extracellular polysaccharides (IEPs), and intracellular polysaccharides (IPs). First, an aliquot of 200 µL of the master biofilm suspension was centrifuged at 10,000 *g* for 5 min at 4°C to determine the SEPs from the supernatant.[17] Second, the resulting pellet was treated with 200 µL of 1M NaOH, homogenized and centrifuged to obtain the IEPs from the supernatant. Third, to the pellet resulting from the previous extraction, 200 µL of 1M NaOH were added for 15 min at 100ºC in a digital block heater (SBD 120-2, Select Bioproducts, Wembley, Perth, Australia), and the pellet was centrifuged at 10,000 *g* for 5 min at 4°C to obtain the IPs concentration. Each fraction of the supernatants was treated with three volumes of cold absolute ethanol and incubated for 30 min at -20ºC. Samples were centrifuged and the resulting pellet was washed with cold 70% ethanol and centrifuged again. The pellet obtained was resuspended in 1M NaOH and the total concentration of carbohydrates contained in each fraction was calculated using the phenol-sulfuric acid method[16] in a microplate reader (ELx800, Biotek, Winooski, Vermont, USA) at 490 nm. Results were standardized according to the dry weight of the biofilm and expressed as percentage of polysaccharides/mg of biomass.

c. *Total proteins*: From the biofilm suspension, 50 µL were treated with 2 M NaOH and incubated at 100°C for 15 min. The suspension was centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant used to determine the concentration of total proteins through the Bradford assay (Bradford reagent, Merck, Darmstadt, Germany) in a microplate reader (ELx800, Biotek, Winooski, USA) at 595 nm.

d. *Bacterial cell counting*: Using serial dilutions from the biofilm suspensions in 0.9% NaCl (v/v), 10 µL was drop-plated on BHI agar plates in duplicate, incubated anaerobically for 24 h at 37°C and colonies counted from the dilution that allowed visualization of distinctively isolated colonies. Counting was corrected by the dilution factor and expressed as CFU/mL.

Polarized light microscope

Enamel slabs were exposed a biofilms de *S. mutans* during 8 h of initial formation under 10% sucrose to observe enamel demineralization (caries-like lesion formation). After the experimental phase, the slabs were washed with 0.9% NaCl to remove the biofilms. An area of the slabs was covered with nail polish as sound enamel control. The slabs were observed at 5x magnification through a polarized light microscope (Primotech, Zeiss, Oberkochen, Germany). This assay was carried out just to portrait the extent of enamel demineralization in an image, and was not done with all the slabs.

Statistical analysis

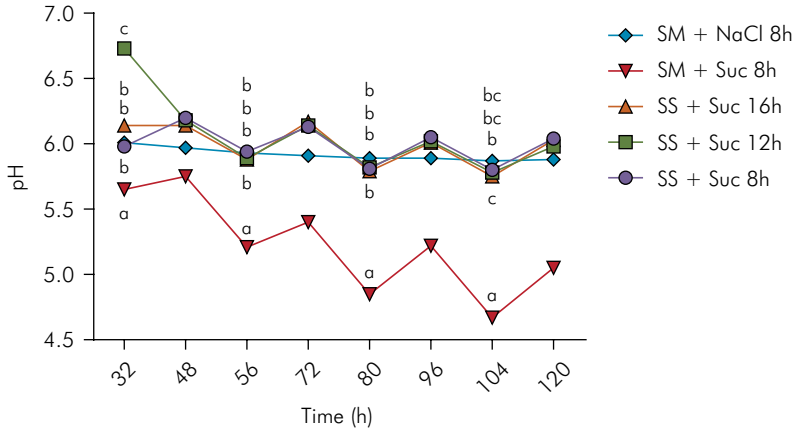
Data were analyzed with the SPSS 15.0 statistical software for Windows (SPSS Inc., Chicago, Illinois, USA). The dependent variables demineralization, biomass, protein content and polysaccharides were subjected to a multiple comparison analysis of a factor through ANOVA followed by the Tukey's post-hoc test. Differences were considered significant if *p*-value was lower than 0.05.

Results

Biofilms of *S. mutans* exposed to sucrose elicited higher acidogenicity in the culture medium

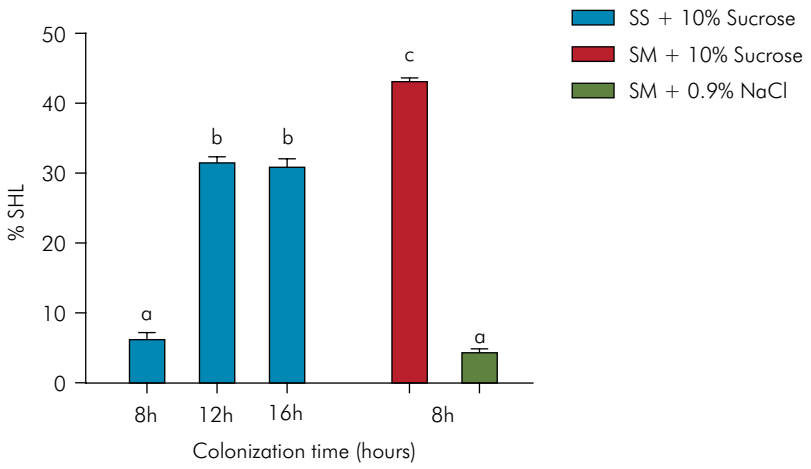
in comparison to the biofilms of *S. sanguinis* (Figure 1). The highest acidogenicity of culture medium measured as pH values was 4.67 ± 0.04 in the *S. mutans* biofilms in sucrose after 8 h of initial biofilm formation (after 104 h had elapsed), while *S. sanguinis* biofilms showed 5.80 ± 0.08, 5.78 ± 0.09 and 5.74 ± 0.09, after 8, 12 and 16 h of initial biofilm formation, respectively. Acidogenicity from *S. sanguinis* biofilms showed a time-dependent behavior, where longer initial biofilm formation time induced higher acidogenicity ($p < 0.05$). The caries-negative control of *S. mutans* biofilms exposed to 0.9% NaCl induced similar acidogenicity (6.01 ± 0.06) when compared to *S. sanguinis* biofilms exposed to 10% sucrose 3 times per day in the first condition.

Biofilms of *S. sanguinis* with an initial biofilm formation time of 12 h and 16 h showed higher



The biofilms were initially allowed to develop for different times on dental enamel and were treated with 10% sucrose solution, three times a day, for 5 min each time, for 5 days [120 h]. The pH of the culture medium was measured at 32 h and then twice a day. *S. mutans* biofilms in 10% sucrose solution and 0.9% NaCl represent caries-positive and caries-negative control, respectively. Each point represents the average of two independent experiments conducted three times [n = 6]. The error bars show the SD. Different letters represent statistically significant differences [$p < 0.05$].

Figure 1. Acidogenicity of *S. sanguinis* biofilms at different times since initial dental enamel biofilm formation times.

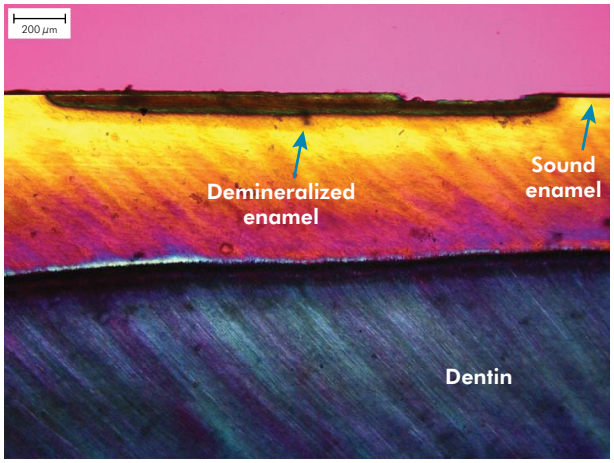


For each enamel slab covered by *S. sanguinis* biofilms, surface hardness [SH] was measured before and after each experimental phase as a % of surface hardness loss [%SHL]. *S. mutans* biofilms in 10% sucrose and 0.9% NaCl represent caries-positive and caries-negative control, respectively. Each bar represents the average of two independent experiments conducted three times [n = 6]. The error bars show the SD. Different letters in each bar represent statistically significant differences [$p < 0.05$].

Figure 2. Demineralization of dental enamel after the formation of *S. sanguinis* biofilms at different times since initial biofilm formation.

demineralization (31.49 ± 2.03% and 32.20 ± 4.0%, respectively) than those with an initial biofilm formation time for only 8 h (6.18 ± 2.46%) ($p < 0.05$) (Figure 2). In contrast, *S. mutans* enamel adherence for 8 h under sucrose resulted in significantly more demineralization (43.09 ± 1.21%) than any of the conditions for *S. sanguinis* ($p < 0.05$). Figure 3 serves to appreciate the extent of enamel slabs demineralization exposed to *S. mutans* biofilms during 8 h of initial formation under sucrose.

Biofilms formed on enamel in sucrose by *S. mutans*, showed significantly higher biomass and protein content ($p < 0.05$) than any of the *S. sanguinis* biofilms (Table 1). Although not statistically significant, the soluble extracellular fraction of polysaccharides (SEPs) was higher at 12 h (0.98 ± 0.2%/mg biomass) and 16 h (1.48 ± 0.38%/mg biomass) of initial adherence than at 8 h (0.79 ± 0.4%/mg biomass) in *S. sanguinis* biofilms and slightly lower than in *S. mutans* biofilms (1.81 ± 0.26%/mg biomass). The IEPs fraction produced by *S. mutans* in sucrose (0.68 ± 0.09%/mg biomass) was slightly higher than any of the *S. sanguinis* biofilms ($p > 0.05$). The IPs did not reveal differences either in biofilms of *S. sanguinis* cultured with different initial biofilm formation times or in *S. mutans* biofilms ($p > 0.05$). Biofilms of *S. sanguinis* formed with different initial adherence times, however, showed no significant differences in protein content, or biomass after 16 h of initial biofilm formation ($p > 0.05$).



Representative sample of enamel slabs exposed to *S. mutans* biofilms during 8 h of initial formation under 10% sucrose. Light blue arrows indicate enamel demineralization [caries-like lesion formation] in the center of the slab and the covered edge used as sound enamel control. The picture was obtained through a polarized light microscope at 5x magnification.

Figure 3. Enamel demineralization and lesion formation.

Bacterial viable counts (Table 2) showed that there was a significantly higher number of viable cells of *S. mutans* during the 8-hour biofilm formation in sucrose ($p<0.05$), than those of *S. sanguinis* cells at any time of biofilm formation, either in sucrose or in NaCl. *S. sanguinis* cells did not show differences among all the time point and culture conditions tested ($p > 0.05$).

Discussion

S. sanguinis is considered a commensal and an early colonizer of the enamel, apparently forming less cariogenic biofilms than *S. mutans*, which has led to consider it as associated with caries-free surfaces.[5,6] Acidogenicity data seem to support this concept, whereby *S. sanguinis* biofilms induced lower pH drops than *S. mutans* (Figure 1). One potential way to

Table 1. Biofilm properties after exposure of dental enamel to different biofilm formation times in presence of sucrose for both bacteria and NaCl in *S. mutans*. Mean (SD), n = 6.

Species	Dental enamel biofilm formation time	Biomass	Proteins	SEPs	IEPs	IPs
		(mg)	(mg/mg biomass)	(%/mg biomass)	(%/mg biomass)	(%/mg biomass)
Streptococcus sanguinis	8 h	0.67 (0.26)^{a}	0.04 (0.03)^{a}	0.79 (0.40)^{ab}	0.31 (0.21)^{a}	0.18 (0.05)^{a}
	12 h	0.83 (0.41)^{a}	0.08 (0.04)^{ab}	0.98 (0.20)^{ac}	0.37 (0.12)^{ab}	0.13 (0.07)^{a}
	16 h	1.08 (0.49)^{a}	0.09 (0.03)^{ab}	1.48 (0.38)^{bc}	0.38 (0.22)^{ab}	0.12 (0.09)^{a}
Streptococcus mutans	8 h	3.42 (0.49)^{b}	0.24 (0.09)^{c}	1.81 (0.26)^{c}	0.68 (0.09)^{b}	0.28 (0.03)^{a}
Streptococcus mutans	8 h (0.9% NaCl)	0.83 (0.41)^{a}	0.13 (0.05)^{b}	0.61 (0.30)^{a}	0.22 (0.06)^{a}	0.15 (0.01)^{a}

SEPs: soluble extracellular polysaccharides, IEPs: insoluble extracellular polysaccharides, IPs: intracellular polysaccharides. Different letters represented as superscript across columns correspond to statistically significant differences ($p < 0.05$).

Table 2. Bacterial counts after exposure of dental enamel to different biofilm formation times in the presence/absence of sucrose for *S. sanguinis* and *S. mutans*.

Condition	Biofilm formation times (h)	Viable cells (UFC/mL)*	
		S. sanguinis	*S. mutans*
0.9% NaCl	8	$^{a}4.49x10^{6} \pm 4.57x10^{5}$	$^{a}1.78x10^{7} \pm 2.13x10^{6}$
	12	$^{a}4.13x10^{6} \pm 1.23x10^{6}$	
	16	$^{a}3.00x10^{6} \pm 3.77x10^{5}$	
10% Sucrose	8	$^{a}4.35x10^{6} \pm 5.31x10^{5}$	$^{b}2.99x10^{10} \pm 2.52x10^{9}$
	12	$^{a}4.73x10^{6} \pm 2.79x10^{6}$	
	16	$^{a}6.11x10^{6} \pm 1.49x10^{6}$	

*Mean ± SD, n = 6. Different letters represented as superscript across columns correspond to statistically significant differences ($p < 0.05$).

explain these results is that *S. sanguinis* is part of the arginolytic microbiota. Arginolytic bacteria are capable of metabolizing protein components from the medium and using them as substrate (such as urea or arginine) for the production of ammonia (NH_3) through urease enzyme or the arginine deiminase system (ADS), respectively.[22] NH_3 production allows pH homeostasis in the oral biofilm, neutralizing acids since the ADS can be activated in slightly acidic conditions.[23] This metabolic ability may explain why *S. sanguinis* is less acidogenic compared with *S. mutans* biofilms, even though both were frequently exposed to sucrose.

Although the pH in our model did not reach pH 4, an inhibitory effect of low pH in *S. sanguinis* biofilms may also explain the lower biofilm formation [biomass, protein, polysaccharides and viable cells] observed here, as compared to *S. mutans* cells in sucrose. *S. mutans* metabolic machinery is less sensitive to lower pH allowing it to produce acids and survive under low pH stress more effectively.[24] Regardless of the potential explanations above, it may well be that sucrose fermentation rate of *S. sanguinis* is just slower than in *S. mutans*, affecting the speed that acids are produced and probably cell division, as well. It has also been described that in excess of sugar, non-mutans streptococci bacteria such as *S. sanguinis*, produce acids, lowering the pH to 5-6 and under these conditions acquire greater acid tolerance by inducing stress proteins such as heat shock proteins (Hsp60-like), which could protect intracellular proteins from acid damage.[25] On the other hand, acid tolerance response (ATR) in *S. mutans* is efficiently triggered by low pH in the environment. Mediated by the expression of a membrane-bound, proton-translocating F1-F0 ATPase, the role of ATR is to maintain an alkaline cytosol, compared to the acidic biofilm environmental conditions.[26] Unlike *S. mutans*, *S. sanguinis* does not seem to trigger similar ATR against acidic environment. Indeed, when compared to *Streptococcus gordonii*, another commensal *Streptococcus*, acid tolerance exhibited by *S. sanguinis* at pH 5.5 seems to be less robust.[27] This can also explain why acid production in *S. sanguinis* upon sucrose exposure is weak and does not seem to increase dramatically over time.

Initial colonization time is important during early dental biofilm formation.[28] Likewise, the time in which early colonizing bacteria adhere to intact enamel may result in different cariogenic properties of the biofilms.[14] Consistent with other *in vitro* caries models,[29] sucrose exposure to *S. mutans* biofilms induced %SHL of about 45% (Figure 2). Values for *S. sanguinis*, however, were lower and dependent on initial adherence time. While *S. sanguinis* biofilms formed initially for 8 h showed similar demineralization compared to the caries-negative control of *S. mutans* biofilms exposed to 0.9% NaCl, *S. sanguinis* biofilms colonizing the enamel for 12 and 16 h showed higher demineralization than those exposed for 8 h ($p < 0.05$), significantly lower than *S. mutans* (Figure 2). Preliminary experiments showed that only 8 h for *S. mutans* were enough to reach similar cariogenicity than *S. sanguinis* at 16 and for that reason, we only used a single time point for *S. mutans*. In an *in vivo* situation, early colonizers adhere to the tooth within the first 4 to 8 h of biofilm formation, mainly comprising bacteria from the *Streptococcus*

genus.[12] It is important to underline that we did not detect significant differences in the number of cells among the three time points (Table 2). This is explained because *S. sanguinis* growth diminish at pH 6 and is slower than at pH 7, but it is not inhibited, without differences in the production of lactic acid.[27] So, we believe that in the initial phase, although *S. sanguinis* is producing acids as part of its basal metabolism, it is also activating the metabolic machinery that will allow it to adapt and create an appropriate alkaline microenvironment for maturation. Hence, greater demineralization resulting from longer *S. sanguinis* adherence to dental enamel in the presence of sucrose, might be better attributable to more acid retention within the biofilm. When allowed to colonize for longer periods of time and in the presence of sucrose, *S. sanguinis* seems to change from a protective commensal microorganism of the dental biofilm to a slightly cariogenic bacterium. Indeed, metabolism analysis of oral bacteria shows that upon an excess of sugars, bacteria from the genus *Streptococcus*, such as *S. sanguinis*, increase the intracellular level of fructose 1,6-bis phosphate, resulting in an activation of pyruvate kinase, accelerating glycolysis and pyruvate production.[30]

Since no significant cell counts were observed in any of the biofilms exposed to 10% sucrose, it is reasonable to speculate that *S. sanguinis* devotes energy to cope with acids produced by the exposure to sucrose rather than proliferating. However, the amount of acid produced *in vivo* might not be enough to induce a shift in the demineralization/remineralization cycle towards caries development since saliva has a remineralizing ability that was not modeled in our approach, but it is currently investigated in more complex polymicrobial biofilm experiments. In polymicrobial diseases, like caries, the resident microbiota certainly should not be considered pathogenic. Yet, under certain environmental conditions, including an excess of sugars, some of the typically considered commensal microorganisms may behave as pathobionts.[9]. Given that *S. sanguinis* cannot achieve comparable demineralization or acid production as *S. mutans*, our data appear to confirm its previously reported commensal nature. Besides colonizing earlier and more abundantly, *S. sanguinis* is key in facilitating subsequent biofilm formation by other bacteria of the dental biofilm and in shaping the ecology of the dental biofilm. The reader is referred to a recent review on *S. sanguinis*, where the authors describe the molecular mechanism of commensalism, which help understanding its role in dental health.[8]

Regarding biochemical and physical biofilm properties, relevant to the virulence of biofilms (Table 1), the amount of biomass formed by *S. mutans* in sucrose was higher than *S. sanguinis* biofilms, and *S. sanguinis* biofilms formed for 16 h showed higher biomass (although not significant) than those allowed to form for 8 h and 12 h (Table 1). When biofilms begin to structure, the synthesis of proteins and enzymes essential for biofilm formation increases, and therefore also biomass and polysaccharides.[4] *S. sanguinis* biofilms formed for 12 h and 16 h, a slight increase of total proteins was observed, but the concentration observed in *S. mutans* biofilms formed for 8 h was much higher, which also coincides with higher biomass quantity.

S. mutans efficiently produces glucan polymers from sucrose, which act as a powerful virulence factor[31] since they are part of the extracellular matrix and they strengthen adherence to dental tissues. In addition, in this matrix the organic acids produced by fermentation are stored, thus a concentration of acid is maintained in contact with the tooth surface.[32] The caries-associated *S. mutans* produces large quantities of glucans by three types of glycosyltransferases: GtfB (produces water-insoluble glucans), GtfC (produces water-soluble and -insoluble glucans) and GtfD (synthesizes water-soluble glucans).[32] GTFs activity in *S. sanguinis* is restricted, with poor glucan production (only GtfS produces water-soluble glucans) and biofilm formation.[33] *S. sanguinis* has a more limited enzymatic machinery for IEPs production, which explains the lower values reached, when compared to *S. mutans*. In fact, we have recently shown that in a dual-species biofilm between *S. mutans* and *S. sanguinis* under sucrose exposure, GTFs expression is dependent on the dynamics of adherence. Thus, there was a downregulation of the GTFs genes during simultaneous biofilm formation, but an upregulation of *gtfB*, *gtfC* and *gtfD* when *S. sanguinis* was the first colonizer and an upregulation of *S. sanguinis*

gtfP (encoding GtfS) when *S. mutans* was the initial colonizer.[34] Based on the biomass data and under the conditions tested here, *S. sanguinis* could become only moderately cariogenic if allowed to colonize for longer periods and frequently exposed to sucrose. Caries has been reported in the absence of cariogenic *S. mutans* and our findings might explain why other species are able to produce acid through fermentation, which could be responsible for this observation.[35] Indeed, and although it produces less acid than *S. mutans*, *S. sanguinis* could contribute to the pool of acids formed by the entire dental biofilm resulting in demineralization. Yet, few studies have considered the cariogenic potential of *S. mutans* when growing with other bacteria under a cariogenic environment. It has been reported that when growing together with the commensal *S. gordonii*, similar to *S. sanguinis*, and in the presence of a non or high-sucrose diet, *S. mutans* was capable of adhering to enamel and outcompeting *S. gordonii*.[36] Moreover, carious lesions were more abundantly induced by *S. mutans* than by *S. gordonii*. The predominant effect of *S. mutans* occurred regardless of the diet, the strain of *S. mutans* or the timing of the inoculation of the bacteria. These results may be similar to what we found here. Not even extending biofilm formation time by *S. sanguinis* is possible to match cariogenic potential of *S. mutans*.

In a complex biofilm, the dynamics of acid formation is much more intricated, so these experiments maybe an oversimplification of the actual behavior of the dental biofilm to model the actual biofilm dynamics. Despite the experimental nature of these experiments and their inherent limitations, however, the results must be considered a proof-of-principle of the role of an abiotic factor, such as diet (in this case excess of sugars) on the cariogenic potential of the commensal *S. sanguinis* derived from a longer initial biofilm formation time. Thus, considering the results obtained, *S. sanguinis* shows a certain cariogenic potential, albeit lower than *S. mutans* when exposed to high concentrations and frequency of sugars.

Conclusions

Increasing the time of initial enamel biofilm formation by *S. sanguinis*, at the times evaluated in this study and in the presence of sucrose, appears to enhance demineralization and affects the properties of *S. sanguinis* biofilms. Although less cariogenic than *S. mutans*, *S. sanguinis* growing as monospecies biofilms show a cariogenic potential, mainly reflected in enhanced demineralization, when frequently exposed to sucrose.

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