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Streptococcus mutans and *Streptococcus sanguinis* Expression of Competition-Related Genes, Under Sucrose

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Dental caries · Dual-species biofilm · Competition · Sucrose · Gene expression

Abstract
Streptococcus mutans synthesizes 3 glucosyltransferases (Gtfs) associated with cariogenic biofilms, while commensal *Streptococcus sanguinis* produces only one; *gtfP* and hydrogen peroxide (H_2O_2) by SpxB. The aim was to test the hypothesis that under a sucrose-induced cariogenic challenge, the expression of competition-related genes is differentially regulated depending on whether *S. sanguinis* or *S. mutans* primarily colonize enamel. Dual-species biofilms of *S. sanguinis* and *S. mutans* were formed under different colonization sequences on enamel slabs and exposed to 10% sucrose for 5 min, 3×/day for 5 days. Biofilms were analyzed for the transcriptional response of competition-related genes encoding *gtfB*, *gtfC*, and *gtfD* for *S. mutans* and *gtfP* and *spxB* for *S. sanguinis*. In addition, acidogenicity (pH) and viable cells in each of the conditions were determined. For all the genes, a downregulation was observed during simultaneous colonization by both bacterial species. In contrast, *gtfB* was upregulated when *S. sanguinis* was the first colonizer ($p < 0.05$). Both *gtfC* and *gtfD* were upregulated during sequential inoculation with *S. sanguinis* as the first colonizer. An eleven-fold upregulation of *gtfP* was observed in biofilms with *S. mutans* as initial colonizer ($p < 0.05$), with a moderate increase in *spxB* expression. The lowest pH values and viable cells of *S. sanguinis* were observed when *S. mutans* first colonized the enamel slabs, compared to the other conditions ($p < 0.05$). Demanding sucrose-challenged oral environment requires increased expression of virulence traits to effectively compete and thrive in the dental biofilm, especially when the competitor has already colonized the ecological niche.

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Introduction

Caries is a disease caused by changes in bacterial ecology [Simón-Soro and Mira, 2015]. Understanding the molecular mechanisms involved in disease development requires an ecological-based approach taking into consideration that dental biofilm formation is a multispecies process [Takahashi and Nyvad, 2011]. This investigation explores the competition among oral species within the dental biofilm using a dual-species model representing the interplay

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between cariogenic and commensal species. *Streptococcus* is the most prevalent genus present in saliva from children with different caries status and also in caries-free people [Gomar-Vercher et al., 2014]. From a microbiological standpoint, carious lesions occur as a result of an ecological imbalance imposed by frequent carbohydrate consumption [Fejerskov, 2004], recently defined as a polymicrobial dysbiosis [Simón-Soro and Mira, 2015]. If the extended caries ecological hypothesis is used as a conceptual framework [Takahashi and Nyvad, 2008], non-mutans streptococci and *Actinomyces* should be considered responsible for maintaining the dynamic stability of the hard dental tissues in health (dynamic stability stage). Upon frequent carbohydrate exposure with subsequent acid production, an adaptation process occurs, whereby certain species, including "low-pH-tolerating" non-mutans bacteria prevail, shifting the demineralization-remineralization process from a net mineral gain to a net mineral loss (acidogenic stage). In this unbalanced acidic environment, mutans streptococci and other aciduric microorganisms induce the onset of carious lesions through a net mineral loss (aciduric stage) [Takahashi and Nyvad, 2016].

Among frequently consumed carbohydrates, sucrose has been widely considered the most cariogenic [Anderson et al., 2009]. Sucrose metabolization by bacteria of the dental biofilm results in organic acid production, which acidifies the local environment. This low pH promotes the growth and predominance of acidophilic and acid-tolerant bacteria by displacing those associated with oral health (reviewed in [Marsh and Zaura, 2017]). It has been recently reported that enamel demineralization induced by sucrose follows a frequency-dependent pattern, seemingly by modification of bacterial virulence traits, including polysaccharide and acid production as well as biomass accumulation [Diaz-Garrido et al., 2016]. These changes seem to occur at a fairly low sucrose concentration, ranging from 5 to 10% [Aires et al., 2006].

Although not etiological according to modern concepts, *Streptococcus mutans* is usually recovered from cavitated lesions and has undoubtedly a high cariogenic potential [Kleinberg, 2002; Loesche, 1986]. Despite the fact that advances in high throughput "omics" technologies suggest that *S. mutans* is poorly represented in the bacterial community [Simon-Soro et al., 2014], this bacterium has a remarkable virulence machinery, including the production and excretion of organic acids (such as lactic acid) and bacteriocins, for example, mutacins I and IV [Merritt and Qi, 2012]. Furthermore, *S. mutans* synthesizes 3 glucosyltransferases (Gtfs) enzymes (GtfB, GtfC, and GtfD) to form extra and intracellular polysaccharides (glucans) from sucrose. GtfB synthesizes water-insoluble glucans; GtfC produces a mix of soluble and water-insoluble glucans and GtfD synthesizes water-soluble glucans [Koo et al., 2009]. Gtfs support bacterial adhesion to dental enamel creating conditions for the formation of a cariogenic biofilm [Bowen and Koo, 2011]. Furthermore, glucans synthesized by Gtfs serve as a carbohydrate source during periods of nutrient limitation (reviewed in [Paes Leme et al., 2006]).

Like *S. mutans* in caries, *Streptococcus sanguinis* is considered a model commensal bacterium, associated with the establishment of healthy dental biofilms [Kreth et al., 2017]. *S. sanguinis* has been typically isolated from caries-free children and adults [Caufield et al., 2000; Ge et al., 2008; Giacaman et al., 2015b]. *S. sanguinis* can produce GtfS (encoded by *gtfP*) [Yoshida et al., 2014] and hydrogen peroxide (H_2O_2), produced by the pyruvate oxidase SpxB [Carlsson et al., 1983]. H_2O_2 production by *S. sanguinis* acts as an antimicrobial compound, interfering with *S. mutans* colonization of the teeth [Kreth et al., 2008]. Competition strategies have been described between *S. sanguinis* and *S. mutans* to predominate within the dental biofilm under cariogenic or health-related conditions [Kreth et al., 2008]. How *S. mutans* and *S. sanguinis* virulence genes are differentially expressed during a simulated sucrose-rich cariogenic environment with sequential colonization has not been described. Understanding the dynamics of the competition within the simulated cariogenic biofilm would greatly increase our mechanistic understanding of disease development and eventually lead to novel therapeutic approaches. This article presents an experimental dual-species caries model, based on sequential inoculation and sucrose exposure to determine the expression of *S. mutans gtfB*, *gtfC* and *gtfD*, and *S. sanguinis gtfP* and *spxB*.

Materials and Methods

Bacterial Strains and Media

S. mutans UA159 (Sm) and *S. sanguinis* SK36 (Ss) were grown in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany) at 37°C and 10% CO_2. Stock solutions of 1 mM glucose and 10% sucrose were prepared. Sucrose solution contains 10 g sucrose, 1.28 mM $CaCO_3$, 0.74 mM K_2HPO_4 and 0.023 ppm NaF per 100 mL [Ccahuana-Vasquez and Cury, 2010]. Both solutions were sterilized by autoclaving at 110 °C for 15 min.

Experimental Design

Single and dual-species biofilms of *S. mutans* and *S. sanguinis* were cultured using an in vitro caries model, previously reported [Ccahuana-Vasquez and Cury, 2010], with modifications [Gia-

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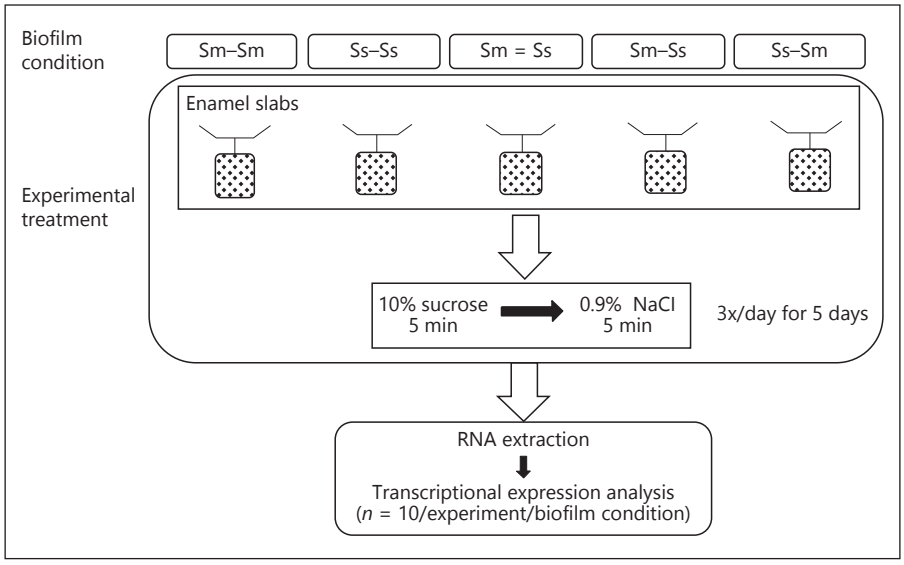

Fig. 1. Schematic diagram of the study design. Single and dual-species biofilms of *S. mutans* and *S. sanguinis* were tested using an in vitro caries model. Biofilms were formed under different colonization sequences on dental enamel slabs: Sm followed by Ss (Sm–Ss), Ss followed by Sm (Ss–Sm), Sm and Ss inoculated at the same time (Sm = Ss) and the single-species controls. Mature biofilms were exposed to 10% sucrose for 5 min 3 times per day at defined times followed by an exposure in 0.9% NaCl for the same amount of time. Sucrose treatment was repeated for 5 days and the assays were carried out in 10 replicates and in 2 independent experiments (n = 20). Biofilms were recovered from the enamel slabs for RNA extraction and transcriptional expression analysis of competition-related genes of both bacteria.

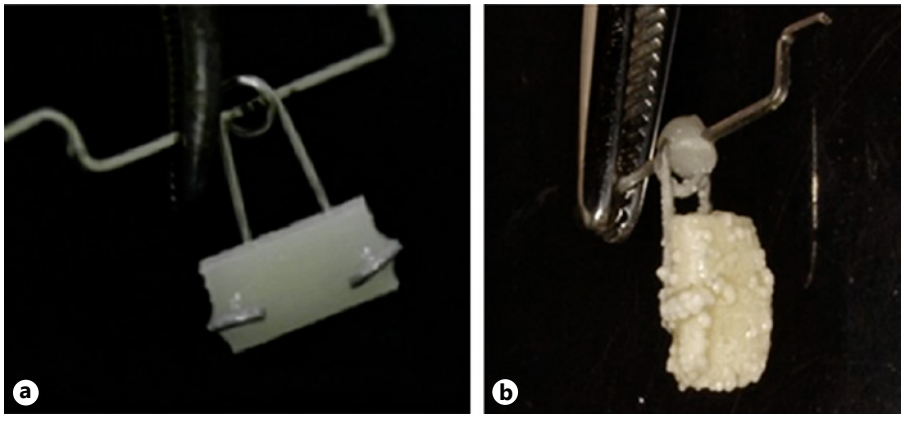

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Fig. 2. Enamel slabs used as substrate for biofilm formation. **a** Saliva-coated enamel slab before sucrose exposure held by steel appliances. **b** Enamel slab with a mature biofilm formed after 5 days of sucrose exposure.

caman et al., 2015a]. Biofilms were formed under 5 different colonization sequences on bovine dental enamel slabs: (1) Sm followed by Ss (Sm–Ss), (2) Ss followed by Sm (Ss–Sm), (3) Sm and Ss inoculated at the same time (Sm = Ss) and the single-species controls (4) Sm followed by Sm (Sm–Sm), and (5) Ss followed by Ss (Ss–Ss). After inoculation, biofilms were allowed to mature in the presence of 0.1 mM glucose for 32 h followed by the cariogenic exposure with 10% sucrose, 3 times per day for 5 min over a 5-day period. Biofilms were recovered from the enamel slabs to analyze transcriptional expression of competition-related genes of both bacteria (Fig. 1).

Enamel Slabs

Slabs were obtained from bovine incisors, previously disinfected with a 5% NaOCl solution and stored in 0.9% NaCl (w/v) until use, not longer than 30 days. Enamel slabs were prepared and sterilized by autoclaving [Diaz-Garrido et al., 2016]. To allow the formation of an acquired pellicle-like structure supporting bacterial adhesion, sterile slabs were covered for 30 min at 37 °C with ultrafiltered (0.22 µm) human saliva from a healthy volunteer fasting for 12 h. Saliva was mixed with AB adsorption buffer (50 mM KCl, 1 mM KPO_4 [0.35 mM K_2HPO_4 and 0.65 mM KH_2PO_4], 1 mM $CaCl_2{\cdot}2\ H_20$, 0.1 mM $MgCl_2{\cdot}6\ H_20$, pH 6.5) 1:1 (v/v) [Lemos et al., 2010] and PMSF protease inhibitor 1:100 (v/v) 0.1 M [Koo et al., 2003]. Slabs were suspended into the wells of a 24-well cell culture plate (Costar®, Corning, NY, USA) by means of a metal holder made with orthodontic wire (Fig. 2a).

Single and Dual-Species Biofilms S. mutans *and* S. sanguinis

To reactivate frozen stocks, bacteria were cultured in BHI supplemented with 1% glucose and incubated at 37 °C and 10% CO_2. Saliva-covered slabs were inoculated with BHI supplemented with 10% sucrose, and bacteria adjusted to an OD_{600} of 0.1 (that is equivalent to 10^{3-4} CFU/mL) in a total volume of 50 mL. To promote biofilm formation, cultures were initially grown for 16 h at 37 °C and 10% CO_2 followed by growth in BHI supplemented with 0.1 mM glucose for 16 h to allow biofilm maturation, which simulates the basal glucose concentration in saliva [Ccahuana-Vasquez and Cury, 2010].

Cariogenic Challenges with Sucrose

Slabs with mature biofilms were exposed to 10% sucrose for 5 min 3 times per day at defined times according to [Diaz-Garrido et al., 2016; Ccahuana-Vasquez and Cury, 2010]. Control treat-

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Table 1. Primers used for transcriptional analysis by real-time PCR

Gene	Primer	5′ to 3′ Sequence	Reference
gtfB	gtfBF gtfBR	AGCCGAAAGTTGGTATCGTCC TGACGCTGTGTTTCTTGGCTC	[Ding et al., 2014]
gtfC	gtfCF gtfCR	TTCCGTCCCTTATTGATGACATG AATTGAAGCGGACTGGTTGCT	[Ding et al., 2014]
gtfD	gtfDF gtfDR	CAGGCAGCCAACGCATTAA AGCCCTCGCTCATCATAAGC	[Banu et al., 2010]
gtfP	gtfPF gtfPR	TCGTCTATCCTTGCTTTATTCG ACGGTCTGCACTTCACTATCA	[Moraes et al., 2014]
spxB	spxBF spxBR	AATTCGGCGGCTCAATCG AAGGATAGCAAGGAATGGAGTG	[Zheng et al., 2011]
16S	16SF 16SR	AAGCAACGCGAAGAACCTTA GTCTCGCTAGAGTGCCCAAC	[Zheng et al., 2011]

F, forward; R, reverse.

ments were done with 0.9% NaCl for the same amount of time. After exposure, biofilms were washed with 0.9% NaCl and returned to the previous culture plate with BHI supplemented with 0.1 mM glucose. Growth media was replaced twice per day, before the first and after the last sucrose exposure. Sucrose treatment was repeated for 5 days and the assays were carried out in 10 replicates and in 2 independent experiments ($n = 20$).

Biofilm Acidogenicity

A pH-cyclic model was used, where biofilms were sucrose-challenged 3 times per day and kept under basal glucose concentrations overnight [Aires et al., 2008]. To assess acid production, medium pH was measured twice per day, after each medium change with a microelectrode (Orion 910500, Thermo Scientific, Waltham, MA, USA) coupled to a pH-meter (Orion Star A211, Thermo Scientific).

Bacterial Counts

By the end of each experiment, a 100-µL aliquot of the biofilm suspension was serially diluted up to $1{:}10^8$ (v/v) in 0.9% NaCl. Fifty microliter of each dilution was seeded in triplicate on Prusian Blue agar (PA) for *S. sanguinis* and Mitis Salivarius Bacitracin agar for *S. mutans.* Plates were incubated at 37 °C and 10% CO_2 (Multigas Incubator, Panasonic, MCO-19M, Osaka, Japan) for 48 h. Total colony counting for both species was carried out from the dilution that allowed the best visualization of individual and separate colonies. Data obtained were corrected by the dilution factor and expressed as CFU/mL.

RNA Extraction

Single and dual-species biofilms were recovered from enamel slabs (Fig. 2b) by shear force using a Maxi Mix II 37600 Mixer (Thermolyne, Iowa, USA) in 1 mL of 0.9% NaCl for 1 min at maximal speed. The resulting biofilm suspension was centrifuged at 8,000 *g* for 8 min at 4 °C and stored in RNAlater (Sigma, St. Louis, MO, USA), according to manufacturer's instructions until further analysis. RNA was isolated as described previously [Cury et al., 2008].

cDNA Synthesis and Real-Time Polymerase Chain Reaction

Total RNA was treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, USA) to remove genomic DNA. The RNA concentration was determined spectrophotometrically in a Multi-Modal Synergy H1 reader (Biotek, Winooski, VT, USA). RNA (1–3 µg) was reverse transcribed into cDNA with random hexamer primers (Thermo Scientific) and RevertAid reverse transcriptase (Thermo Scientific), according to manufacturer's instructions. Preparations without reverse transcriptase were used as a control of cDNA synthesis reactions and to evaluate contamination with genomic DNA. Transcriptional analysis of the *spxB* and *gtfP* of *S. sanguinis* and *gtfB*, *gtfC* and *gtfD* of *S. mutans* was performed with gene-specific primers listed in Table 1. The quantitative polymerase chain reaction (qPCR) reactions were performed in a real-time thermocycler StepOnePlus (Applied Biosystems, Foster, CA, USA) using Maxima SYBR Green/Rox qPCR kit (Thermo Scientific), following instructions from the manufacturer. Ct values obtained under each condition were normalized to the respective value for the control gene (16S rRNA) and subsequently expressed as function of the control condition (NaCl) using the ΔΔCt algorithm [Livak and Schmittgen, 2001]. The relative expression of the genes mentioned in the control condition (NaCl) was arbitrarily assigned with a value of 1.

Statistical Analysis

Data were analyzed using Minitab version 17 software (Minitab Inc, State College, PA, USA). Student *t* test was used to compare the difference of the means of the gene expression under each condition, relative to the control condition (NaCl). The non-parametric Wilcoxon test was used to compare the median expression of each gene under all conditions (as group), compared to the control

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Table 2. pH values at different times (hours) for each biofilm condition

Hours	Ss–Ss	Sm–Sm	Sm = Ss	Ss–Sm	Sm–Ss
40	5.84 (0.20)[b]	5.23 (0.03)[a]	5.76 (0.04)[b]	5.88 (0.05)[b]	5.18 (0.15)[a]
64	5.80 (0.17)[c]	4.84 (0.13)[a]	5.52 (0.05)[b]	5.69 (0.08)[b, c]	4.89 (0.11)[a]
88	5.68 (0.03)[d]	4.55 (0.13)[a]	5.22 (0.11)[c]	5.56 (0.14)[d]	4.74 (0.08)[b]
112	5.73 (0.03)[d]	4.35 (0.04)[a]	5.11 (0.08)[c]	5.50 (0.08)[e]	4.51 (0.04)[b]

Mean (SD). Different letters in superscript (a–e), represent statistical differences among the inoculation sequences, across each time point ($p < 0.05$).

Table 3. Bacterial counts of *S. sanguinis* and *S. mutans* at the end of the experiment in each biofilm condition

Viable cells, CFU/mL	Ss–Ss	Sm–Sm	Sm = Ss	Ss–Sm	Sm–Ss
S. sanguinis	9.53E+05±1.22E+05[a]	–	9.47E+05±5.88E+05[a]	6.07E+05±9.02E+04[a]	1.07E+02±5.03E+01[b]
S. mutans	–	9.53E+08±1.22E+08[a]	1.32E+08±3.82E+07[b]	4.87E+07±2.04E+07[b]	6.00E+08±2.00E+07[c]

Mean ± SD. Different letters in superscript (a–c), represent statistical differences ($p < 0.05$).

condition. The pH and cell count dependent variables were analyzed using the analysis of variance test. Differences were considered significant when the p value was lower than 0.05.

Results

pH as a Measure of Biofilm Acidogenicity

When *S. mutans* was the enamel colonizer (Sm–Ss), biofilms showed a more pronounced pH decrease over time, as compared with the other groups ($p < 0.05$), except with the Sm–Sm control biofilm, which was more acidogenic from 88 h ($p < 0.05$; Table 2). When both species were inoculated at the same time (Sm = Ss), biofilm pH was intermediate between the pH values of Sm–Sm and Sm–Ss, and those from Ss–Ss and Ss–Sm ($p < 0.05$). Indeed, Ss–Ss and Ss–Sm conditions showed the highest pH of all the experimental settings ($p < 0.05$). Only at the end of the experiment, the Ss–Ss biofilm showed slightly higher pH than the dual-species biofilm of Ss–Sm ($p < 0.05$).

Viable Cells

Bacterial counts were carried out at the end of the experimental phase for each biofilm condition (Table 3). *S. mutans* counts were significantly reduced when *S. sanguinis* was the first colonizer or when both species were inoculated at the same time ($p < 0.05$), without differences between them ($p > 0.05$). Conversely, *S. sanguinis* was reduced only when *S. mutans* was the first to colonize the enamel ($p < 0.05$).

Transcriptional Analysis of S. mutans *Virulence Genes in Single and Dual-Species Biofilms with Sucrose Exposure*

Expression of genes encoding for the 3 Gtfs, *gtfB*, *gtfC*, and *gtfD* were analyzed and compared with or without sucrose exposure, in the single and dual-species biofilms. When *S. mutans gtfB* expression in all inoculation sequences was compared against their respective control biofilm, in the absence of sucrose (Fig. 3a), a 0.33- and 0.78-fold decrease was observed in the single-species condition (Sm–Sm) and the simultaneous inoculation condition (Sm = Ss) respectively ($p < 0.05$). No differences were detected for the Sm–Ss, where *S. mutans* was the first colonizer ($p > 0.05$). Conversely, a 4.1-fold increase was observed for *gtfB* when *S. sanguinis* was the first colonizer (Ss–Sm).

The expression of *gtfC* (Fig. 3b) and *gtfD* (Fig. 3c) followed a similar trend when compared with the expression of *gftB*, under same conditions described above. The downregulation was significant when *S. mutans* colonized first (Sm–Ss) or at the same time as *S. sanguinis* (Sm = Ss; $p < 0.05$). Expression of both genes increased significantly when *S. sanguinis* was the first colonizer of the enamel slabs (Ss–Sm; $p < 0.05$), with a 4.7- for *gtfC* and a 2.8-fold increase for *gtfD*, when compared to the control biofilms without sucrose.

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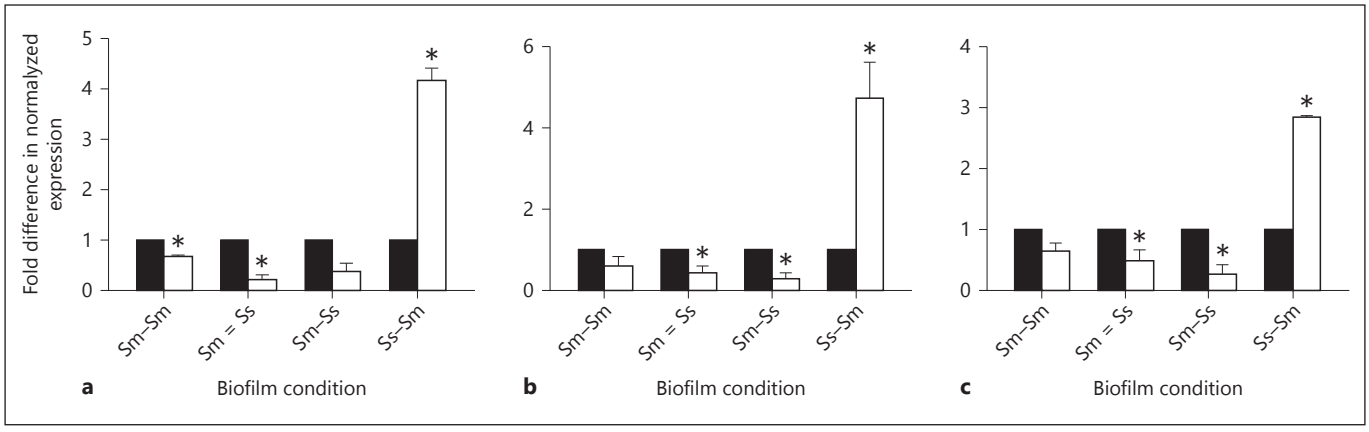

Fig. 3. Transcriptional expression of analyzed genes in *S. mutans,* in single- and dual-species biofilms. Relative expression of the genes in the control condition (NaCl) was arbitrarily set with a value of 1. **a** *gtfB* gene; (**b**) *gtfC* gene; (**c**) *gtfD* gene. Expression was normalized using 16S rRNA gene expression. Bars represent the means of the genes expression and the error bars are the SD of the analysis of the real time-PCR performed at least twice in triplicate of the 2 independent experiments (biofilms) in each condition. The Wilcoxon non-parametric test was used to compare medians of the expression of each gene in all conditions (colored bars) relative to the control condition NaCl (grey bars). * Significant difference ($p < 0.05$) respect to control.

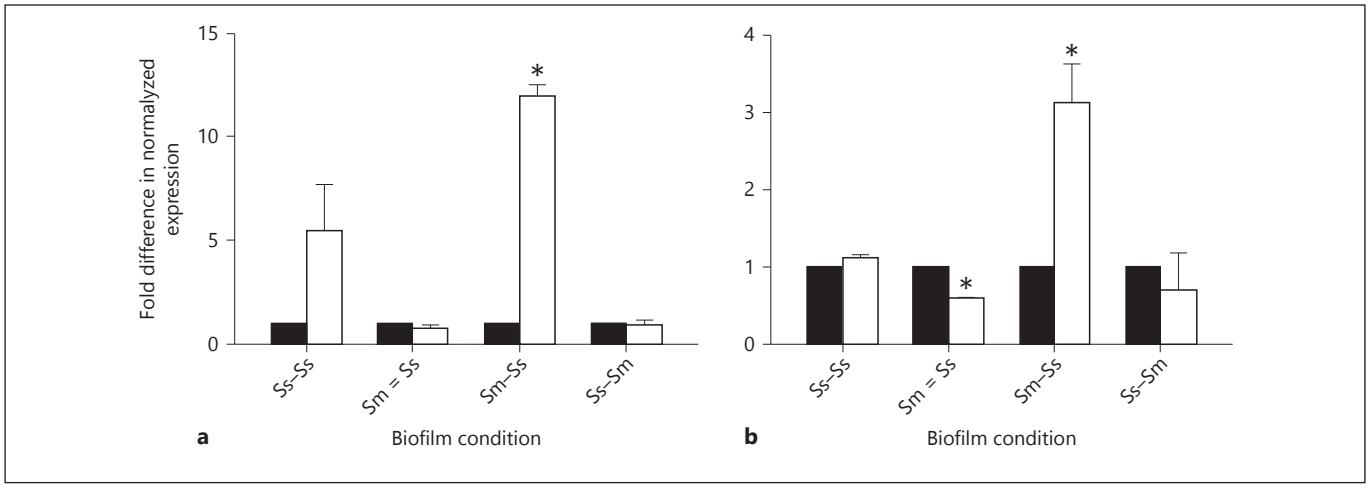

Fig. 4. Transcriptional expression of analyzed genes in *S. sanguinis,* in single- and dual-species biofilms. Relative expression of the genes in the control condition (NaCl) was arbitrarily set with a value of 1. **a** *gtfP* gene and (**b**) *spxB* gene. Expression was normalized using 16S rRNA gene expression. Bars represent the means of the genes expression and the error bars are the SD of the analysis of the real time-PCR performed at least twice times in triplicate of the 2 independent experiments (biofilms) in each condition. The Wilcoxon non-parametric test was used to compare medians of the expression of each gene in all conditions (colored bars) relative to the control condition NaCl (grey bars). * Significant difference ($p < 0.05$) respect to control.

S. sanguinis *Gene Expression in Single and Dual-Species Biofilms Exposed to Sucrose*

The expression of *gtfP* (Fig. 4a) and *spxB* (Fig. 4b) was assessed after 5 days of exposure to 10% sucrose in the caries model, with the no-sucrose condition for all the inoculation sequences. When *S. mutans* colonized and adhered to dental enamel prior to *S. sanguinis* (Sm–Ss), the expression of the *gtfP* showed more than an 11-fold increase compared to the caries-negative control biofilm without sucrose ($p < 0.05$). No differences in the expression were detected in *gtfP* under Sm = Ss or Ss–Sm conditions, compared to their respective negative controls ($p > 0.05$).

The expression of *spxB*, involved in H_2O_2 production, was also determined (Fig. 4b). Compared to the caries-negative control condition, without sucrose, no differen-

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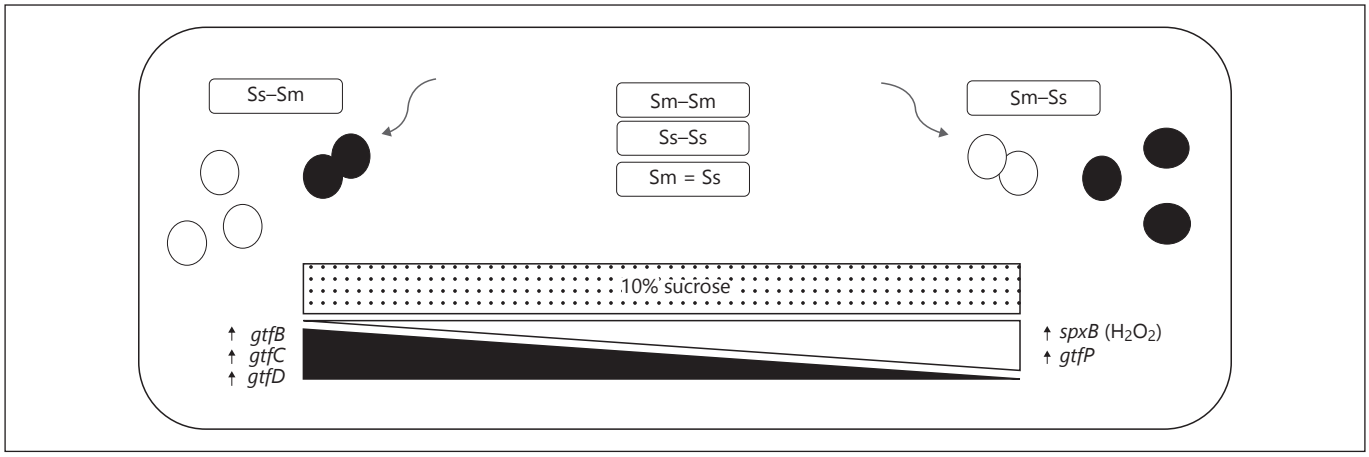

Fig. 5. Competition model between *S. mutans* and *S. sanguinis* according to experimental design. Different timings of colonization and biofilm formation on enamel under excess sugar simulating a cariogenic environment. Only when both bacteria act as invasor microorganism overexpress competition-related genes (*gtfB*, *-C* and *-D* of Sm and *gtfP*, *spxB* of Ss) to lead in the biofilm. In the Sm–Sm, Ss–Ss, and Sm = Ss conditions, such expression is not significantly greater with respect to the biofilms caries-negative controls. White circles: Ss; black circles: Sm. Arrows indicate the entry of the invading microorganism.

tial expression of the gene was detected either in single-species biofilms (Ss–Ss) or in the dual-species biofilms when *S. sanguinis* was the initial colonizer (Ss–Sm). Although not statistically significant, a trend for a lower expression of *spxB* was observed when *S. sanguinis* was the initial colonizer (Ss–Sm biofilm). On the contrary, a 3.2-fold upregulation of *spxB* ($p < 0.05$) was detected when *S. mutans* was the initial colonizer, followed by the incorporation of *S. sanguinis* in the dual-species biofilm (Sm–Ss), suggesting a more pronounced competitive behavior. When both species colonized the enamel at the same time (Sm = Ss), a 0.4-fold downregulation was detected ($p < 0.05$).

Discussion

The dynamics of the biological interactions (commensalism, synergism, competition) among microorganisms that form dental biofilms are not well understood. It is even less known how they respond, at the molecular level, to variations in the local oral microenvironment. Ecological changes could positively or negatively affect the dynamic interplay in the biofilm, leading to health or disease, such as caries or periodontal diseases. Since carbohydrates are considered the main etiological factor for caries [Sheiham and James, 2015], these nutrients will influence the interactions within the dental biofilm leading to the ecological shift or dysbiosis described in caries [Simón-Soro and Mira, 2015]. Indeed, this study was carried out with that theoretical framework in mind.

We analyzed the transcriptional response of important competitive genes of an early dental biofilm colonizer, *S. sanguinis* as well as known virulence genes of the cariogenic *S. mutans*, during different timings of colonization and biofilm formation on enamel. In addition, the pH values and viable cells of both bacteria were determined in each biofilm condition tested. A validated experimental model was used simulating high frequency of sucrose exposure to induce a cariogenic environment.

When biofilm acidogenicity was assessed, there was a pH drop to values lower than 4.5 for the Sm–Ss and Sm–Sm biofilms, which confirms the highly cariogenic, acid- and exopolysaccharide-producing traits of *S. mutans* in response to a high sucrose concentration in the medium [Bowen and Koo, 2011]. Likewise, the high viable cell counting for *S. mutans* and the lower for *S. sanguinis* was consistent with the pH data. On the other hand, when *S. sanguinis* colonizes first or forms single-species biofilms, higher pH values (>5.8) and higher cell counts were observed. The latter confirms the idea of *S. sanguinis* as being an arginolytic commensal, able to maintain an alkaline pH within the oral microenvironment by ammonia production from urea or arginine, using the arginine deiminase system, activated by environmental pH values slightly acidic [Burne and Marquis, 2000; Nascimento et al., 2014].

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Figure 3 depicts gene expression of *S. mutans* Gtfs; *gtfB*, -*C* and -*D* and Figure 4 shows *S. sanguinis* genes, *gtfP* and *spxB*. In general, the expression of all *S. mutans gtf* genes decreased when *S. mutans* was the first colonizer or when both species were inoculated at the same time, relative to each of their respective control biofilms without sucrose. These results were consistent with a previous study [Wen et al., 2010], where a decreased expression of *gtfB,* though not significant, was observed in simultaneously inoculated dual-species biofilms of *S. mutans* and *S. sanguinis*, compared to the single-species biofilm of *S. mutans*. Conversely, there was a significant increase of these genes when *S. sanguinis* was the first colonizer (Ss–Sm). The final pH for this biofilm, however, was ≥5.5 (critical pH for enamel demineralization), with higher *S. mutans* than *S. sanguinis* counts ($p < 0.05$).

On the other hand, there was an increase of *gtfP* and *spxB* expression, when *S. mutans* was the initial biofilm colonizer and *S. sanguinis*, though with lower cell counts, intended to colonize the already occupied ecological niche. These results suggest that the invading microorganism must express its main virulence genes involved in biofilm formation and competition to produce insoluble and soluble glucans to remain adhered and to avoid displacement by the colonizing species.

The presence of the disaccharide sucrose in the media allows *S. mutans* to produce these complex exopolysaccharides (glucans), which in turn facilitate biofilm adhesion through glucan-binding proteins (GBPs) [Klein et al., 2015]. In fact, it has been reported that the expression of the *gtfB* and *gtfC* are induced under the influx of fermentable sugars or under an acidogenic microenvironment [Li and Burne, 2001]. Although Gtfs are key to form biofilms, in our study, the genes encoding for the enzymes failed to show upregulation when *S. mutans* was the first colonizer and during single-species condition (Sm–Sm; Fig. 3a–b), unlike when *S. mutans* acted as the late colonizer (Ss–Sm). Due to the latter, it is reasonable to speculate that the sole exposure to sucrose is not enough to induce the expression of the genes considered in this study. Indeed, the combined effect of sucrose exposure and the dynamics of colonization seem to play a key role in promoting gene expression to compete for the ecological niche. Hence, only when *S. mutans* invades an already colonized niche by *S. sanguinis* (Ss–Sm), these genes are upregulated (Fig. 3). Conversely, when *S. mutans* is the first colonizer ([Sm–Sm], [Sm–Ss], [Sm = Ss]), sucrose appears required for *S. mutans* to proliferate and express virulence traits, such as exopolysaccharides, organic acids and adhesion proteins, to promote the formation of an acidic and dense biofilm, selective for acid-tolerant species. Once formed, *S. mutans* does not need to overexpress these genes as showed in these experiments.

It is interesting to mention that in the study by [Shemesh et al., 2007], the authors showed that *gtfB* and *gtfC* were upregulated in response to sucrose but downregulated in the simultaneous presence of glucose and sucrose. These findings suggest that glucose affects the expression of *S. mutans* biofilm genes. In our model, the medium used contained glucose only, without sucrose and when sucrose was presented to the biofilms, no glucose was present. Therefore, no simultaneous presence of glucose and sucrose was present in our experiments. The latter confirms the idea that the low *gtfs* expression in the biofilms where *S. mutans* was already colonizing the enamel seems to be due to the order of colonization rather than to the presence of sucrose. It is likely that the dynamics of enamel colonization is more important to determine gene expression than the type of the available nutrients. Alternatively, when *S. mutans* is the early colonizer, cells might prioritize competition against the invading bacteria, rather than forming polysaccharides. Moreover, *S. mutans* can produce antimicrobial peptides, named mutacins, which are potent repressors of other bacterial species [Merritt and Qi, 2012].

The fact that cell counts and *gtfP* and *spxB* genes from *S. sanguinis* were not affected by *S. mutans* invasion of already formed *S. sanguinis* biofilms (Ss–Sm) was quite surprising. It appears that once *S. sanguinis* first colonizes the biofilm, cells are not capable of mounting a defense against the invading *S. mutans* cells, at least under cariogenic environmental conditions, like those created for our experiments. These findings are also reflected by clinical observation where *S. sanguinis* seems to prevail in low-risk children [Ge et al., 2008] and adults [Giacaman et al., 2015b] but tends to be outnumbered or outcompeted in high-risk individuals who have frequent carbohydrate exposure, among other risk factors. From a microbiological standpoint, it is reasonable to speculate that *S. mutans* invasion under frequent sucrose exposures would create environmental pressure and stress (low pH due to increased acid production) for less aciduric species, like *S. sanguinis*. In this context, *S. sanguinis* cells already adhered to the enamel may halt biofilm expansion. It has been reported that *S. sanguinis* compromises viability under acidic conditions, in addition to inhibiting enzymes of the glycolytic pathway [Takahashi et al., 1997]. Conversely, *S. sanguinis* tend to form polysaccharides and H_2O_2 when they need to attach onto established *S. mutans* biofilms with a significant increase in *gtfP* and

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spxB expression (Fig. 4a, b; Sm–Ss condition), though with low cell counts, as compared to those of *S. mutans* (Table 3). This is further supported by the observation that no significant increase of *gtfP* and *spxB* expression was detected with the single-species biofilms (Ss–Ss).

The *gtfs* genes from *S. mutans* and *S. sanguinis* and *spxB* from the latter would be overexpressed only when each of them must act as the invading species, competing against the virulent metabolic products from the antagonist species, that is, glucans and H_2O_2. In this context, during the period in which the colonizing species has not seen the invading bacterium, there is no need to significantly express their genes and a basal level of expression would be enough to thrive (Fig. 5). In Figure 5, a graphical representation of the competition model between *S. mutans* and *S. sanguinis* discussed here is depicted.

Taken together, our results suggest that colonization and fitness in the dental biofilm constitute a dynamic process that involves several environmental conditions, including nutrient availability and colonization sequence. These experiments were designed as a proof-of-principle and a model for the complex interactions that occur in the actual dental biofilm. Gene regulation upon changing environments observed here confirms the idea of caries as being a non-infectious polymicrobial dysbiosis [Sanz et al., 2017]. It is clear that diet-influenced local environments promote ecological switches in the dental biofilm and deserved a deeper look, not only on the effect of sucrose, but also to the role of the other nutrients to which the dental biofilm is permanently exposed as recently discussed [Giacaman, 2017]. Doubtlessly, more research is required to better understand the highly intricate relationships among bacterial species in the dental consortium, not only on the biofilm species composition but also on the metabolic activities exerted by its members [Takahashi, 2015].

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Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Author Contributions

R.A.G. coordinated and conducted this investigation and is the PI of the major project that gave rise to it; N.D.-G. performed the biofilm assays and contributed ideas to the paper; C.P.L. performed the biofilm assays, RNA extractions, Real Time-PCR analysis and drafted the manuscript; J.K. critically revised and contributed ideas. All of the authors discussed the results, read, and approved the final manuscript.

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