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Effect of Sucrose on the Behavior of a Dual-Species Biofilm of *Candida albicans* and *Streptococcus sanguinis*

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Abstract: *Candida albicans* is present in oral health and has been linked with dental caries. So far, the behavior of the yeast in the presence of *Streptococcus sanguinis* has not been tested. The aim was to determine the effect of excess sugar as a cariogenic environment on the biological and physico-chemical parameters of single-species (*C. albicans*) and dual-species biofilms of *C. albicans* and *S. sanguinis*. An in vitro caries model was used. The biofilms were exposed to 10% sucrose or 0.9% NaCl for 5 min 3x/day/5 days. Viable cells, hyphal quantification, SEM, biofilm pH, H_2O_2 quantification and surface microhardness of enamel slabs were determined. Microbial viability and the number of hyphae were higher for sucrose exposure, in the dual-species biofilms induced by excess sugar and coexistence between the two microorganisms. Sucrose-exposed biofilms showed a progressive pH drop over the time course. H_2O_2 production was about 6 times higher for the dual-species biofilms. Enamel demineralization was higher in *C. albicans* biofilm, suggesting an inhibitory effect due to *S. sanguinis*. Excess sugar increases the pathogenic potential of *C. albicans* biofilm. In dual-species biofilms, *S. sanguinis* prevents pronounced drops in pH, decreasing demineralization and stimulates the development of hyphae in the yeast.

Keywords: Excess sugar; *Candida albicans*; *Streptococcus sanguinis*; single-species biofilm; dual-species biofilm

1. Introduction

Abundant communities of hundreds of species comprising complex biofilms can be found in the oral cavity of humans [1,2]. To date, the expanded Human Oral Microbiome database (*e*HOMD; www.homd.org) informs 774 oral bacterial species, of which only 58% are officially named. Still scarcely understood, species within these biofilms may act as commensal or as competitive microorganisms, thus showing antagonistic or synergistic interactions. Given the complexity of the oral microbiome, control mechanisms should take place among the component microorganisms to preserve an equilibrated condition of eubiosis [3]. Until now, it has been very difficult to study the variety of interactions between the different microorganisms that form part of the dental biofilm in health and disease oral, i.e., dental caries. Species of the genus *Streptococcus* colonize the oral cavity (about 20%) at an early stage and are the pioneers in the formation of dental biofilm, a favorable oral health condition [3,4]. *S. sanguinis* is an early colonizer in dental biofilm formation. It is associated with the surface of caries-free teeth and is a native inhabitant microorganism of the mouth [4,5]. This bacterium produces and secretes hydrogen peroxide (H_2O_2) as an antimicrobial compound to compete with other oral streptococci [5,6]. It has been described that H_2O_2 production by commensal streptococci (as *S. sanguinis*) is the principal determinant, bacteriostatic at low concentrations and capable of preventing the growth of bacteria such as *S. mutans*, a cariogenic microorganism [7],

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reviewed in [8]. Hence, H_2O_2 is proposed as a key component for the maintenance of oral ecology associated with healthy conditions [9]. On the other hand, *Candida* spp. are also common commensal microorganisms in the oral cavity, with approximately 50-60% prevalence in healthy people [10,11]. Under conditions of disturbance of the oral microenvironment or dysbiosis (as dental caries or periodontitis), *Candida* spp. become opportunistic and pathogenic, condition known as pathobiont, where resident and commensal microorganisms may turn pathogenic and trigger the onset of oral diseases [12]. Even though *Candida albicans* is the most frequently isolated fungi from the oral cavity, its interaction with commensal microorganisms remains poorly understood. *Candida* spp. have been linked with dental caries, being isolated with a high frequency (mainly *C. albicans*) from children with caries lesions compared to caries-free children [13,14]. However, it is still uncertain whether the increase in *C. albicans* counts involves it with dental caries or wether it is due to factors directly implicated in the beginning of the caries process or the yeast plays an active role in promoting this disease. To date, the biological interaction between *S. sanguinis* and *C. albicans* has not been tested, although both are part of the dental biofilm. In this study, we will use *S. sanguinis*, which is directly correlated with oral health and presents some features of a model microorganism, to study molecular commensalism [5].

On the other hand, the emerging and scarce knowledge on the oral fungal microbiota, including *Candida* spp., has limited the studies on the interactions between bacteria-fungus and the role they play in health and disease [15,16]. Recently, studies using "omics" technologies have characterized the oral mycobiome in oral health and disease (as dental caries). A marked prevalence in the presence and proportions of *Candida* spp. yeasts was reported [17,18]. *Candida* spp. present several virulence traits that could influence caries development, such as adhesion to tooth surfaces, degradation of extracellular matrix and proteins [19], colonization of the oral mucosa and fermentation of carbohydrates [20] contributing to the acidic environment existing in caries. In this context, the hydroxyapatite, the major component of dental enamel formed by a phosphate and calcium apatite complex, is severely affected [21]. Under this acidic environment (pH<5.5), the enamel, the outermost mineral layer of the tooth, begins to demineralize [22]. Moreover, *C. albicans* changes from yeast-like (ovoid cell) to hypha, a frequently analyzed virulence marker whose morphology prevails when it is part of biofilms [23].

C. albicans co-aggregates with *S. sanguinis* in in vivo and in vitro conditions [24,25]. On the other hand, in the oral cavity, *Candida* spp. is constantly exposed to reactive oxidative species, such as H_2O_2, produced by some bacteria (such as *S. sanguinis*) and immune cells present in the oral cavity [26]. H_2O_2 induces genotoxic and oxidative stress in yeast, promoting hyphal formation [27,28]. However, this yeast is protected through several detoxification systems [29].

Current concepts accept that *C. albicans* is a commensal microorganism under oral health conditions but may transition to a pathobiont when the ecological balance is lost. The specific factors (intrinsic conditions of the oral microenvironment or of the oral microbiota present) that trigger the transition are mostly unknown. This article evaluates the effect of *S. sanguinis* on *C. albicans* in dual-species biofilms regarding biological and physico-chemical parameters, in an in vitro model of caries.

The results of this study may contribute important information to further develop novel strategies for caries control, including fungi, along with bacteria, as a potential therapeutic target.

2. Materials and Methods

Microorganisms and growth conditions: Oral clinical isolate of *Candida albicans* from caries-free preschool children (ICDAS II code = 0), (part of the strain collection of our laboratory) and *S. sanguinis* SK36 (from human dental plaque) were used in this study. The Todd Hewitt Broth medium (THB; BD, MD, USA) was used for individual growth of bacteria pre-cultures with 5% CO_2 for 18 h at 37ºC. Sabouraud-dextrose (SD; BD, MD, USA) broth was used in *C. albicans* aerobic pre-cultures overnight at 37ºC. Yeast Nitrogen Base (YNB; BD, MD, USA) broth supplemented with 0.1 mM glucose or 10% sucrose according to experimental design was used in single- and dual-species biofilms. The study protocol and informed consent (because of the use of human saliva for biofilm formation) were

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approved by the Ethics Committee of the Faculty of Dentistry, University of Chile (certificate Nº5/2022).

Single-species biofilm (*C. albicans*). This model was performed according to [30] with some modifications. That is, the biofilms were formed on saliva-coated enamel slabs (prepared from bovine incisors as described in [31]) as substrates to grow biofilms. Human non-stimulated saliva was obtained and filtered using filters with a pore size of 0.22 µM (Corning, NY, USA), obtained from healthy volunteers fasting for 10 h mixed with 1X AB buffer (50 mM KCl, 1 mM KPO_4 [0.35 mM K_2HPO_4 and 0.65 mM KH_2PO_4], 1 mM $CaCl_2{\cdot}2H_2O$, 0.1 mM $MgCl_2{\cdot}6H_2O$, pH 6.5) and 0.1 M PMSF protease inhibitor (Sigma-Aldrich, MD, USA) was used to form the acquired salivary pellicle on the enamel slabs (sES). The sES were suspended vertically in a 24-well culture plate (Costar®, Corning, NY, USA) by orthodontic wire. sES were inoculated with *C. albica*ns cultures (OD_{600nm} of 0.38, equivalent to 10^7 CFU/mL) in YNB broth supplemented with 10% sucrose solution and incubated for 1.5 h at 37ºC with gentle agitation (to allow the adherence phase of biofilms). Then, 10% fetal bovine serum (FBS; Gibco, NY, USA) (for hyphal formation) was added and incubated for 10 h at 37ºC in microaerophilic conditions. The biofilm was allowed to mature in YNB broth supplemented with 0.1 mM glucose for 18 h in the same conditions as above, with a fresh medium change at 12 h. Subsequently, the experimental design consisted of the biofilms being exposed 5 min, 3 times/day for 5 days to 10% sucrose solution (excess sugar) or 0.9% NaCl (control condition). The treatment was carried out in 6 biological replicates per condition from two independent experiments (n=12).

***C. albicans* and *S. sanguinis* dual-species biofilm.** This model is similar to the single-species biofilms with modifications [32]. After the *C. albicans* biofilms attachment phase, 10% FBS, *S. sanguinis* culture (OD_{600nm} of 0.5, equivalent to 10^7 CFU/mL), THB medium and YNB supplemented with 10% sucrose solution were added and incubated for 10 h at 37°C under microaerophilic conditions. The biofilms were allowed to mature in YNB broth supplemented with 0.1 mM glucose and THB medium for 18 h under the same conditions as above, with a fresh medium change at 12 h. Subsequently, the biofilms were exposed to the same experimental treatment as the single-species biofilms, in 6 biological replicates in 2 independent experiments (n=12).

Biological and physico-chemical parameters (described below) were measured from single- and dual-species biofilms. Additionally, observations of the biofilms were made using Scanning Electron Microscopy (SEM) under the conditions previously indicated.

Biological parameters: A) Viable cells: After 5 days of experimental treatment of the single- and dual-species biofilms, enamel slabs were removed from the wire and transferred into sterile tubes with 1 mL 1X PBS pH 7.4. The biofilms were dispersed with a sonicator with 3 pulses of 30 s at 7 W with 1 min interval on ice. This "master solution" was serially diluted in PBS and seeded in duplicate on THB agar and SD agar (supplemented with chloramphenicol at a final concentration of 20 µg/mL) to obtain CFU/mL of bacteria and yeast, respectively. The plates were incubated aerobically at 37ºC for 48 h for the development of yeast colonies, while quantification of bacterial colonies was performed by incubating the plates in 5% CO_2 at 37ºC for 24 h. Colony quantification was done in 6 biological replicates per condition from 2 independent experiments (n=12). **B) Hyphal quantification:** In single- and dual-species biofilms, this quantification was performed as previously described by [25]. Briefly, 50 µL of master solution of each biofilm were spread on a glass slide and viewed under a light microscope (Standard 20, Carl Zeiss, Jena, Germany) at 40x magnification. Ten microscopic fields per slide were analyzed in duplicate in the 3 biological replicates per condition.

Physico-chemical parameters: A) pH measure in biofilms: pH was determined according to [32] introducing a microelectrode (35811-98 OAKTON, IL, USA) coupled to a pH-meter (ION 700 OAKTON, IL, USA) into each well. Readings were taken twice per day in the spent medium, after overnight incubation and before the last fresh medium change of the day, and during the entire course of the experiment (approx. 142 h). **B) Surface microhardness (enamel demineralization):** It was estimated through the loss of Knoop's surface hardness (SH) in enamel slabs from single- and dual-species biofilms, described in [31]. Briefly, initial surface hardness $(SH)_i$ was evaluated by 3 linear indentations in a Knoop microindenter with a microhardness tester (402 MVD, Wolpert Wilson Instruments, USA), at 50 g/5 s. After the experimental treatment, 3 indentations were made to the

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slabs, adjacent to the initial indentations, considered as final SH $(SH)_f$ (kg/mm^2). The average $(SH)_i$ and $(SH)_f$ were used to calculate the percentage loss of SH (%SHL), interpreted as demineralization: (average $(SH)_i$ − average $(SH)_f$) × 100/$(SH)_i$. **C) H_2O_2 production:** At the end of the assays, the filtered supernatant of the culture medium of each well in single- and dual-species biofilms and the Amplex™ Red kit were used (ThermoFisher Scientific, MA, USA). Briefly, in the presence of peroxidase, Amplex™ Red reagent reacts with H_2O_2 (released from biological samples) in a 1:1 stoichiometry to create a red fluorescent oxidation product, resorufin. Subsequently, H_2O_2 concentration in the biofilm supernatant was calculated according to the supplier's instructions. The quantification was done in 3 biological replicates per condition from 2 independent experiments.

Observation of single- and dual-species biofilms by microscopy: After experimental treatment, single-species (*C. albicans*) biofilms on circular glass discs (1 cm diameter) and dual-species biofilms on the enamel slabs were examined by SEM (JEOL JSM IT300LV; Tokyo, Japan) operated at 20 kV and a 10-mm working distance. Glass discs and enamel slabs with the biofilms were fixed with 2.5% glutaraldehyde and then treated with alcohol in ascending concentration from 50% to 100% which dehydrated the samples. Subsequently, they were subjected to critical point drying and metallized with carbon. The images were the results of general observations of the samples.

Statistical analysis. Data were analyzed using STATA SE software v14. All data were expressed as mean and SD respective or median with ranges, as appropriate. T-test, ANOVA and Tukey's post hoc test were used as well as Wilcoxon test for non-parametric samples. Differences were considered significant if $p<0.05$.

3. Results

3.1. Effect of Sucrose on Biological Parameters in Single- and Dual-Species Biofilms

3.1.1. Cell Viability of *C. albicans* and *S. sanguinis*

When sucrose exposed and unexposed biofilms were assessed, non-significant differences were detected on yeast viability of single-species biofilms with *C. albicans*, with a trend for higher viability under sucrose condition compared to the control (NaCl) ($p>0.05$). Median values were $1.2x10^6$ CFU/mL (range $2.4x10^5$–$5.3x10^6$) and $8.8x10^5$ CFU/mL (range $3.6x10^5$–$2.6x10^6$), respectively (Table 1). For the dual-species biofilms, a significant median difference ($p<0.001$) between the control condition ($1.1x10^6$ CFU/mL, range: $3.0x10^5$–$4.5x10^6$) and the sucrose condition ($1.4x10^6$ CFU/mL, range: $2.7x10^5$–$3.9x10^6$) was observed. The median viability of *S. sanguinis* in the control condition was $8.0x10^7$ (range: $4.0x10^6$–$4.1x10^8$), and in the sucrose condition $1.0x10^8$ (range: $7.0x10^6$–$3.7x10^8$) ($p<0.001$) (Table 1).

Table 1. Viable cell counts of *C. albicans* and *S. sanguinis* in single- and dual-species biofilms in NaCl and sucrose condition.

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Biofilms	Condition	Viable cells (UFC/mL)[#]	
		C. albicans	*S. sanguinis*
Single	0.9% NaCl	$8.8x10^5$ ($3.6x10^5$ - $2.6x10^6$)	---
	10% Sucrose	$1.2x10^6$ ($2.4x10^5$ – $5.3x10^6$)	---
Dual	0.9% NaCl	$1.1x10^6$ ($3.0x10^5$ - $4.5x10^6$)	$8.0x10^7$ ($4.0x10^6$ - $4.1x10^8$)
	10% Sucrose	$^*1.4x10^6$ ($2.7x10^5$ - $3.9x10^6$)	$^*1.0x10^8$ ($7.0x10^6$ - $3.7x10^8$)

[#]Median (ranges) of two independent experiments conducted six times (n=12). Asterisks represent statistically significant differences among experimental conditions. Wilcoxon test, $p<0.05$.

3.1.2. Filamentation of *C. albicans*

In the single-species *C. albicans* biofilms, the control condition showed slightly higher hyphal counts than the sucrose condition, but the differences were not significant ($p>0.05$), with a mean of 5.31 ± 2.56 and 4.42 ± 1.47, respectively (Figure 1). In contrast, the number of hyphae in the dual-species biofilms for both control condition (7.6 ± 2.3) and the sucrose condition (14.9 ± 4.6) showed a significant difference ($p<0.001$). The highest hyphal count was observed in the dual-species biofilms for both experimental conditions ($p<0.001$).

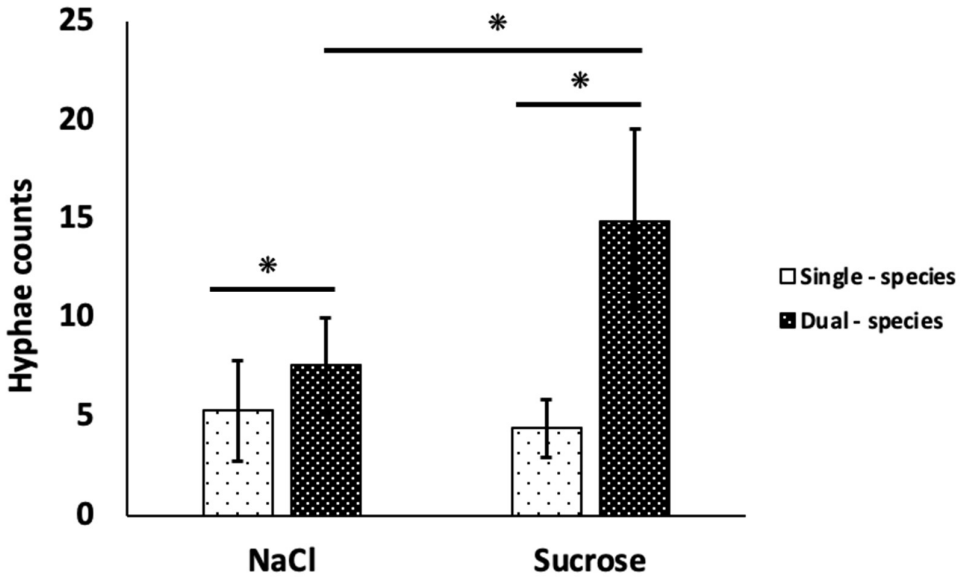

Figure 1. Quantification of *C. albicans* hyphae in single- and dual-species biofilms in both experimental conditions. Bars indicate the average of two independent experiments (n=6). Error lines show standard deviation (SD). ANOVA, $p<0.05$.

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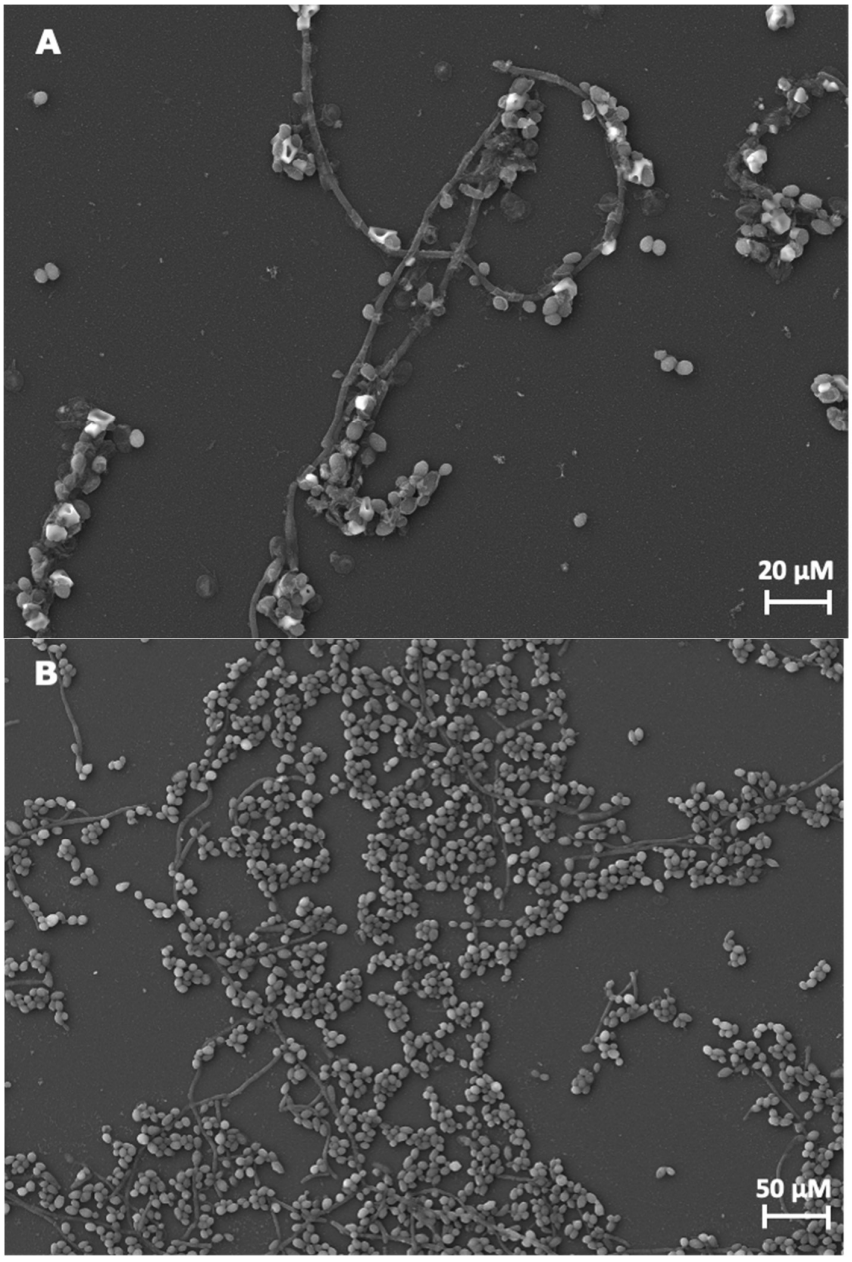
3.1.3. Biofilms Formation

Biofilm formation for both experimental conditions is shown in Figure 2. In the NaCl condition (Figure 2A) of single-species biofilms (*C. albicans*), hyphal structures and their blastospores, as well as some groups of cells forming small aggregates, were observed. Figure 2B depicts sucrose condition with more hyphal and blastospore structures and aggregates are observed, forming a typical biofilm structure.



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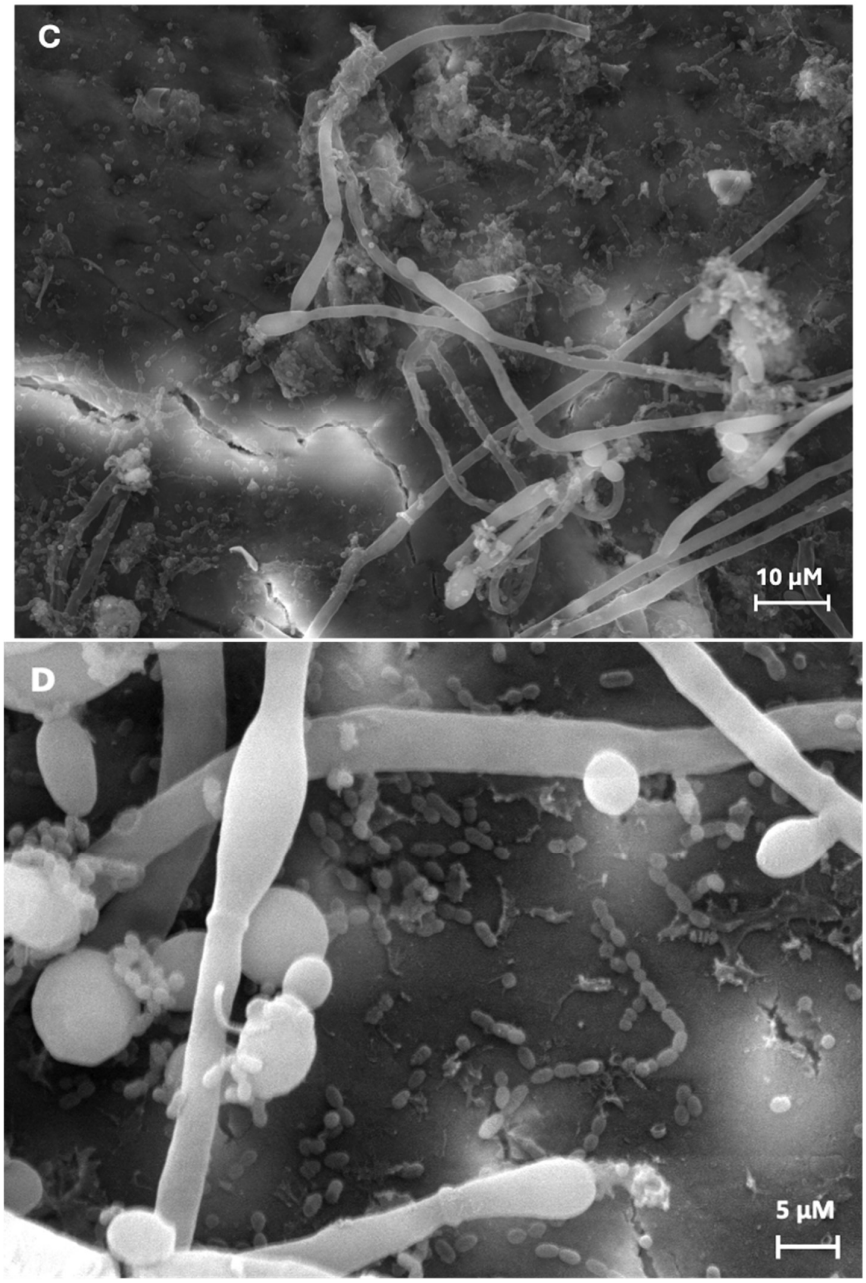

Figure 2. Representative scanning electron microphotographs obtained from the single- and dual-species biofilms in both experimental conditions. A), C) single- (800x) and dual-species (1300x) biofilms in 0.9% NaCl, respectively, and B), D) single- (500x) and dual-species (4500x) biofilms in 10% sucrose, respectively.

In the dual-species biofilms, a greater number of oval forms and yeast hyphae were observed in the sucrose condition (Figure 2D), compared to the control condition (Figure 2C). In addition, proximity (physical interaction) between *C. albicans* and *S. sanguinis* was observed in both conditions.

Noticeably, for both conditions the biofilm extracellular matrix cannot be observed due to the treatment needed for microscopic observation.

3.2. Effect of Sucrose on Physico-Chemical Parameters in Single- and Dual-Species Biofilms

3.2.1. Acidogenicity Measurement in the Biofilms

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Figure 3a shows the mean pH values (representing acidogenicity from the biofilms) of the *C. albicans* single-species biofilms for both experimental conditions. Biofilms exposed to sucrose showed higher acidogenicity compared to biofilms exposed to NaCl. The mean pH value was similar in both conditions until approximately 22 h after the beginning of the experiment ($p>0.05$), and from 30 h to the end of the experiment, a significant decrease in pH was observed in the sucrose condition versus the NaCl condition ($p<0.05$). The highest pH for sucrose-exposed biofilms was 4.18 ± 0.42 at 102 h, while the lowest pH for the control was 5.25 ± 0.33 at 126 h. Figure 3b shows the variation in pH for the dual-species biofilms during the entire course of the experiment (142 h). From 46 h to 142 h, there were significant differences in the pH values between both experimental conditions ($p<0.001$). The lowest pH obtained in the control condition was 6.29 ± 0.14, while the lowest pH obtained in the sucrose condition was 4.98 ± 0.20, both at 126 h.

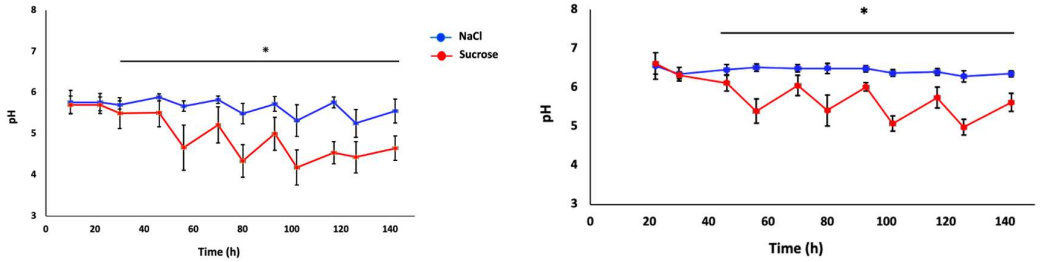

Figure 3. pH variations during the experiment in single-and dual-species biofilms. The blue line represents the control condition (NaCl) and the red line the sucrose condition from two independent experiments (n=12) in *C. albicans* biofilm **(a)**; and *C. albicans*—*S. sanguinis* biofilm **(b)**, respectively. Asterisk represents statistically significant differences among conditions. Error lines show standard deviation (SD). ANOVA and Tukey's post hoc test, $p<0.05$.

3.2.2. Surface Microhardness on Enamel Slabs

Figure 4 shows surface microhardness loss data, which represents enamel demineralization. Enamel slabs exposed to sucrose showed 71.6% ± 9.71, which is slightly higher than the NaCl condition, with 65.96% ± 16.69 ($p>0.05$), in the single-species biofilms. For the dual-species biofilms, sucrose condition showed higher numbers than NaCl, 64.94% ± 13.24 and 53.95% ± 13.60, respectively ($p<0.05$). The lowest demineralization percentages were in the dual-species biofilms for both experimental conditions.

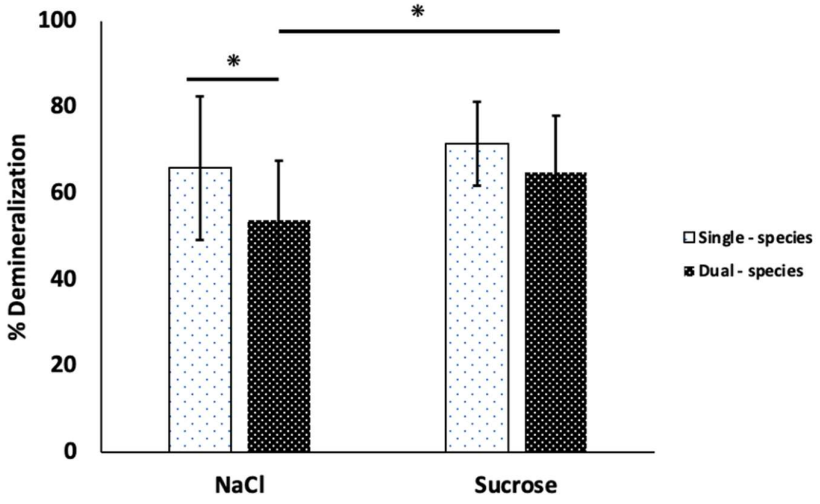

Figure 4. Enamel slabs demineralization of single- and dual-species biofilms after exposure to sucrose and NaCl condition. The surface microhardness of the enamel slabs was measured before

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and after the experimental treatment and interpreted as demineralization percentage. Each bar indicates the average of two independent experiments conducted six times (n=12). Error lines show the standard deviation (SD). Asterisks represent statistically significant differences among conditions ANOVA, $p<0.05$.

3.2.3. H_2O_2 Production in Biofilms

Table 2 shows H_2O_2 quantification for both types of biofilms under the described experimental conditions. For dual-species biofilms, H_2O_2 levels were 3.05 ± 0.99 µM for sucrose and 2.51 ± 0.77 µM for NaCl ($p>0.05$), while in the single-species biofilms they were lower than 0.5 µM. For both experimental conditions, H_2O_2 levels were higher for dual-species than single-species biofilms ($p<0.05$).

Table 2. H_2O_2 quantification in single- and dual-species biofilms under NaCl and sucrose conditions.

Biofilms	Condition	H_2O_2 quantification (µM)[#]
Single	0.9% NaCl	0
	10% Sucrose	0.43 ± 0.33
Dual[*]	0.9% NaCl	2.51 ± 0.77
	10% Sucrose	3.05 ± 0.99

[#]Mean ± SD of two independent experiments conducted three times (n=6). Asterisk represent statistically significant differences among biofilms. T test ($p<0.05$).

4. Discussion

Effect of Sucrose on Biological Parameters Evaluated in Single- and Dual-Species Biofilms

Cell Viability of *C. albicans* and *S. sanguinis*

This study confirmed the ability of *C. albicans* to metabolize sucrose, which favors increased cell viability of dual biofilms. Bezerra et al. [33] observed an increased production of soluble and insoluble extracellular polysaccharides (EPS) in yeast monospecies biofilms subjected to high concentrations of glucose, favoring biofilm formation and increased cell viability. High *Candida* yeast counts have been reported in microenvironments with high sugar concentration and acidic salivary pH. For example, studies have been carried out with oral clinical isolates of *C. albicans* obtained from saliva of patients with type II diabetes mellitus. It was determined that when these isolates were exposed to different concentrations of glucose, they had greater cell viability than those from saliva of non-diabetic subjects [34,35]. High counts of this yeast genus have also been observed in similar microenvironments, such as in the saliva of preschool children with severe caries lesions or ICDAS II code 6 [13]. In the review by Lok et al. [36], it was described that *C. albicans* has the ability to double its viable count only in the presence of sugars such as glucose, fructose and galactose.

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In the same article, it was mentioned that the effect of different carbon sources and the biofilm formation capacity of this yeast were tested, and it was determined that under exposure to sucrose the EPS content, roughness, height and thickness of its biofilm were higher compared to glucose, arabinose and lactate. Interestingly, the article by Xiang et al. [37] determined that clinical oral isolates of *C. albicans* from dental plaque of toddlers with severe childhood caries showed high proteinase activity and acidogenic and aciduric properties. In addition, it was observed that these isolates showed variations in hyphal growth, i.e., some of them had defective hyphal formation and others were hyperfilamentous. Finally, it was observed that mixed biofilms of these yeast isolates with *S. mutans* increased biomass and EPS accumulation versus using reference yeast strains.

In the study by Ev et al. [38], using RNA-seq analysis, it was established that *C. albicans* isolates from active root caries lesions showed overexpression of genes related to metabolic activity, sugar transport, stress tolerance, invasion and pH regulation, compared to isolates from healthy root surfaces, where only genes related to biofilm formation were overexpressed.

Huffines and Scoffield [8] observed that dual-species biofilms of *C. albicans* with *S. parasanguinis* (one of the most abundant commensals) were less robust than those of yeast with *S. mutans*. In addition, *S. parasanguinis* reduced the synergy between them in the biofilm. In the same study, no significant differences were found in the biomass of *S. parasanguinis* in single-species versus dual (with *C. albicans*) and three-species (with *C. albicans* + *S. mutans*) biofilms. In our study, the increase of viable *S. sanguinis* cells in the dual-species biofilm with excess sugar could be explained by the presence of yeast, for its role as a scaffold shaping the structure of polymicrobial biofilms [39], and, by the intrinsic ability of the bacterium to metabolize sugar for cell proliferation [40].

Taken together, both yeast and bacterial viability are promoted induced by excess sugar and when they co-exist in dual-species biofilm.

Hyphal Formation in *C. albicans*

C. albicans is characterized by the fact that it typically grows as a yeast (oval shaped and usually in budding) but can transition to a pseudofilamentous and filamentous form (hyphae) under the influence of environmental cues, such as alterations in temperature, pH and available nutrients. This way of changing from a yeast-like to a hyphal state and vice versa is considered the most pathogenic form of yeast, facilitating the formation of biofilms [41]. It has been determined that in the presence of sugars such as fructose and glucose, the 3 types of cell morphology of this yeast coexist with other sugars (reviewed in [36]).

In their study, do Rosario Palma et al. [25], observed the absence of inhibition of *C. albicans* filamentation. Furthermore, there was transcriptional overexpression (5-fold) of the gene encoding the hyphal wall protein (*HWP1),* involved in yeast adhesion and biofilm formation in the presence of *S. sanguinis* versus monospecies biofilms. Similarly, reviewed in Lohse et al. [42] and [28], pointed to a synergistic interaction between yeast and oral streptococci, such as *S. sanguinis*, promoting bacterial colonization and biofilm formation and, on the other hand, favoring yeast virulence and tissue invasion.

In the study by Morse et al. [43], they observed that hyphal formation increased when *C. albicans* was cultured together with *S. sanguinis, S. gordonii, Actinomyces viscosus* and *A. odontolyticus*. And when adding *Porphyromonas gingivalis* to this microbial consortium, this filamentation capacity was reduced. With this background, we can partly outline that one of the roles of *C. albicans* within these microbial societies is to have a structural function that allows bacterial co-aggregation and biofilm proliferation.

It is worth noting that yeast filamentation was only observed in the dual-species biofilm, where the only modified variable was the presence of *S. sanguinis*, so we can speculate that the presence of the bacterium alone could be inducing hyphal formation. The study by Xu et al. [44] demonstrated that the commensal bacterium *S. oralis* activates the filamentation pathway in *C. albicans*, promoting the interaction between kingdoms (prokaryote—eukaryote) and thus the formation of biofilms. This shows that the morphogenetic trait of the yeast is promoted by the presence of a bacterium alone.

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It has been described that an alkaline pH (pH above 6) induces filamentation in yeast (reviewed in [45]). However, a study by Merino et al. [46] described that this morphological transition also occurs in a pH range from 4.4 to 7.4, which would be promoting the formation of biofilms. Thus, the ability to form hyphae could be a unique trait of the yeast that influences the virulence of biofilms [38].

In vitro studies have reported that H_2O_2 (commercial) induces the morphological transition of *C. albicans* from the yeast form to the hyphal stage [29,47]. Nevertheless, the effect of H_2O_2 biologically produced by *S. sanguinis* on filamentation is unknown. Preliminary studies in our laboratory showed an increase in filamentation of this yeast when exposed to supernatant (filtrate) from *S. sanguinis* cultures in late exponential phase, compared to commercial H_2O_2 and fetal bovine serum (unpublished data). Together with the few existing studies of interactions between *C. albicans* and *S. sanguinis*, the results of this study and unpublished data speculate that the bacteria modulate the pathogenic traits of the yeast.

Effect of Sucrose on Physico-Chemical Parameters Evaluated in Single- and Dual-Species Biofilms

Acidogenicity of the Biofilms

In dual-species biofilms of *C. albicans* with *S. mutans*, Willems et al. [48] found that the pH of the medium was higher than the pH of the single-species biofilm of the bacteria, so that *C. albicans* would be raising the pH in acidogenic microenvironments, as confirmed by Eidt et al. [49]. Despite the above, it should be noted that the articles mentioned are not comparable, because the *C. albicans* strains, the culture medium and the substrate used to form the biofilms are different. However, in our experiments pH values in the *C. albicans* biofilm and in excess sugar were acidic (close to 4.2), whereas for dual-species biofilm these values reached minimum of 5.0, indicating the inhibitory effect of *S. sanguinis* on pH in slightly acidic environments. That is, the bacterium induces the arginine deiminase system (ADS) [50], which generates ammonium from arginine as a substrate present in the proteins of the medium, raising the pH. *C. albicans* cannot produce lactic acid because it lacks the lactate dehydrogenase enzyme. However, it produces other acids, such as malic, pyruvic and acetic acid, which may contribute to the predominant acidic pool, such as a cariogenic condition [20]. This was also confirmed in the study by Bezerra et al. [33], where the pH drop showed a dose-dependent effect with respect to the glucose concentration in the culture medium in monospecies *C. albicans* biofilms. Conversely, monospecies *S. sanguinis* biofilms under similar experimental conditions reached pH values close to 6.0 under excess sugar [51]. Therefore, it is possible to conclude that the presence of *S. sanguinis* in conditions of excess sugar hampers pH drops in dual-species biofilms.

Enamel Slabs Demineralization

One of the pathogenic traits of *Candida* spp. is the production and secretion of extracellular enzymes, such as aspartyl proteases, phospholipases and lipases that are activated at low pH and degrade proteins and extracellular matrix components [19]. It has also been described that *Candida* spp. are able to dissolve dentin and consequently release calcium into the medium [52], but this has not been tested on tooth enamel, which has a higher mineral content than dentin. de Abreu et al. [20] observed that *C. albicans* biofilms formed on bovine enamel slabs coated with a pool of human saliva from preschool children with and without caries showed a higher percentage of demineralization compared to the control, which corresponded to enamel slabs coated with saliva but without the addition of yeast. Also, in the study by Charone et al. [53] using oral clinical isolates of *Candida* spp. from supragingival plaque of HIV-positive children, observed a gradual reduction in the surface microhardness of human tooth enamel from day 1 to day 5 after biofilm formation; it was also observed that *C. albicans* produced calcium release during the experiment. Our study, yeast monospecies biofilms yielded slightly more enamel demineralization, although not significant, in the excess sugar condition than in the control. However, the demineralization percentages were lower in the dual biofilm. This would mean that in excess sugar the presence of *S. sanguinis* inhibits the enamel demineralization preventing a pronounced decrease in pH values.

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Furthermore, in the study by Willems et al. [48], it is shown that biofilms of yeast and *S. mutans* in a glucose medium supplemented with 0.2% sucrose had a more alkaline pH and consequently less calcium release compared to the single-species biofilm of the bacterium. Under these experimental conditions, we can speculate that *C. albicans* has also a role in decreasing cariogenicity in sugar-rich microenvironments and in the presence of an acidogenic bacterium. However, that study did not use enamel slabs for biofilm growth, but hydroxyapatite discs. With the results of our study, we can propose that excess sugar has a greater impact on enamel demineralization in yeast monospecies biofilms than in dual-species biofilms in the presence of the commensal bacterium *S. sanguinis*.

H_2O_2 Production in Biofilms

S. sanguinis produced H_2O_2 in the dual-species biofilm under the conditions of the study and tended to be slightly higher in excess sugar (around 3 μM). In the article by Díaz-Garrido et al. [51], H_2O_2 production was determined to be around 15 μM and 25 μM in a dual-species biofilm of *S. sanguinis* with *S. mutans* and in a single-species *S. sanguinis* biofilm, respectively, using the same in vitro caries model. Competition between these two bacteria has been widely described in the literature [7,32,54]. Thus, this difference in H_2O_2 production levels compared to our results represents that *S. sanguinis* does not need to compete for microenvironment with yeast or it is sufficient to maintain the attenuated virulent traits of *C. albicans*. As for the chemical or metabolic interaction of *S. sanguinis* with *C. albicans*, evidence is scarce. Nevertheless, it is worth mentioning the work by Zheng et al. [55], who found that the carbon catabolite control protein A (CcpA) of *S. sanguinis* regulates the expression of the *spxB* gene, encoding the pyruvate oxidase enzyme (involved in H_2O_2 production). However, this regulation is not influenced by glucose concentration. This relationship may explain why we did not observe significant differences in H_2O_2 production in the control versus sucrose condition.

On the other hand, oral streptococci (*S. oralis*, *S. mitis*, *S. sanguinis* and *S. gordonii*) have been reported to release H_2O_2 into the surrounding environment, which can be inhibitory or toxic to fungal cells, limiting the proliferation of, for example, *Candida* spp. in the host [47]. This fungus has different mechanisms to neutralize ROS and cope with oxidative stress, through the induction of the catalase enzyme gene (*CAT1*) that degrades H_2O_2; the synthesis of antioxidants such as glutathione can also repair oxidative damage to DNA, proteins and lipids (Reviewed in [56]). Additionally, *C. albicans* synthesizes farnesol, a molecule derived from farnesoic acid, which acts in cell-cell communication (quorum sensing), attenuates oxidative stress, and plays a key role in inhibiting bud-to-hyphal transition at high cell densities, without preventing elongation of pre-existing hyphae or affecting population growth [57,58]. Therefore, it can be suggested that H_2O_2 has an inhibitory effect on the viability, filamentation and ability of *C. albicans* to form biofilms with commensal microorganisms, which was not observed in our results. In addition, studies indicate that a neutral or alkaline pH of the medium promotes the formation of filamentous forms of *C. albicans*, while a low pH (present in medium with excess sugar) should inhibit the formation of filamentous forms of *C. albicans* (reviewed in [41]).

Consequently, we can state that excess sugar in a dual-species biofilm of *C. albicans* and *S. sanguinis* has no effect on H_2O_2 production. It remains to be studied whether this chemical produces oxidative stress in yeast.

Candida albicans Role in the Oral Biofilms Structure

The effect of a high-sugar diet on multispecies biofilms has been described in models involving *S. mutans* and *C. albicans*. Bowen et al. [59] indicated that this relationship between kingdoms is due to EPS formation, coadhesion and the role of exoenzymes, such as bacterial Gtfs, in the production of an extracellular matrix (ECM) that allows synergistic interaction between these species. Once these biofilms are formed, both species can exchange metabolites, substrates and growth factors, while continuing to produce exoenzymes and increasing the ECM volume of the biofilm. Thus, it can be deduced that both microorganisms play a structural role within the biofilm. Under oral health conditions, *C. albicans* may interact synergistically with *S. sanguinis* (physically, chemically or

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metabolically), whereby hyphal formation serves as a surface for the adhesion of these bacteria and leads to the establishment of biofilms [39]. In our results, it was observed that the number of yeast cells and hyphae increased considerably under a microenvironment with excess sugar and in the dual-species biofilm. Thus, the only difference with the yeast monospecies biofilm regarding these parameters was the presence of *S. sanguinis*. Unfortunately, SEM images do not show the volume and biomass of the biofilms. Still, in the case of the dual-species biofilm, they show the physical proximity between the two microorganisms. Therefore, using inverted microscopy or CLSM would give us more information about the biovolumes of the biofilms.

While synergism between *S. mutans* and *C. albicans* has been described, there is little evidence regarding oral commensal streptococci and their interaction with yeast. An example of this is the article by Huffines and Scoffield [8], who observed that in multispecies biofilms, *S. parasanguinis* inhibits *C. albicans* adherence to glucans and glucan formation by *S. mutans* by blocking the activity of its Gtfs enzymes, indicating the potential ability of *S. parasanguinis* to attenuate the synergy between these microorganisms and prevent oral dysbiosis. On the other hand, it has been reported that *S. gordonii* cannot co-aggregate with hyphal-defective *C. albicans* isolates. However, the yeast develops robust biofilms with *S. mutans*, independent of the filamentation status [37].

Our results suggest that *S. sanguinis* modulates the virulence of *C. albicans* by affecting hyphal formation and demineralizing capacity. Further studies are needed to understand these interactions and to develop new therapeutic strategies. It is also recommended to investigate how oral microenvironmental factors influence these interactions.

The study and understanding of the biological interactions occurring in oral biofilms should not only be based on bacterial targets. Further research is needed to elucidate how different microorganisms interact and organize in dental biofilm during health or in caries.

5. Conclusions

Excess sugar increases the pathogenic potential of mono-species *C. albicans* biofilms, in cell viability, filamentation and demineralizing capacity. Under conditions of excess sugar, the presence of *S. sanguinis* in dual-species biofilms with an oral clinical isolate of *C. albicans* attenuates abrupt pH drops, decreasing demineralization induced by the yeast. In addition, under this condition, there is increased viability of the bacteria, without affecting H_2O_2 production.

Further studies should deepen these findings, exploring the biochemical and molecular basis of the interaction between *C. albicans* and other commensal oral bacteria, such as *S. sanguinis*. The results suggest potential new strategies for preventing dental caries by harnessing the natural inhibitory effects of *S. sanguinis* on pathogenic yeasts and bacteria. This could lead to the development of probiotic treatments or other microbiome-based therapies.

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Data Availability Statement: All data generated or analyzed during this study are included in this article. Further enquiries for specific data can be directed at the corresponding author.

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References

1. Takahashi, N.; Nyvad, B. The role of bacteria in the caries process: ecological perspectives. *J. Dent. Res.* **2011**, *90*, 294-303. doi: 10.1177/0022034510379602.
2. Simón-Soro, A.; Ren, Zhi.; Krom, B.P.; Hoogenkamp, M.A.; Cabello-Yeves, P.J.; Daniel, S.G.; Bittinger, K.; Tomas, I.; Koo, H.; Mira, A. Polymicrobial aggregates in human saliva build the oral biofilm. *mBio.* **2022**, *13*, e0013122. doi: 10.1128/mbio.00131-22.
3. Marsh, P.D.; Zaura, E. Dental biofilm: ecological interactions in health and disease. *J. Clin. Periodontol.* **2017**, *44*, S12–S22. doi: 10.1111/jcpe.12679.
4. Marsh, P.; Martin, M. Williams, D. In *Oral Microbiology*, 5th ed.; Lewis, MA., Ed.; Edinburgh London New York Oxford Philadelphia St Louis Sydney Toronto, Churchill Livingstone Elsevier, 2009.
5. Kreth, J.; Giacaman, R.A.; Raghavan, R.; Merritt, J. The road less traveled - defining molecular commensalism with *Streptococcus sanguinis*. *Mol. Oral Microbiol.* **2017**, *32, 181–196. doi: 10.1111/omi.12170.*
6. Zhu, L.; Kreth, J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. *Oxid. Med. Cell. Longev.* **2012**, *2012*, 717843. doi: 10.1155/2012/717843.
7. Kreth, J.; Merrit, J.; Shi, W.; Qi, F. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J. Bacteriol.* **2005**, *187*, 7193-7203. doi: 10.1128/JB.187.21.7193-7203.2005.
8. Huffines, J.T.; Scoffield, J.A. Disruption of *Streptococcus mutans* and *Candida albicans* synergy by a commensal streptococcus. *Sci. Rep.* **2020**, *10,* 19661. doi: 10.1038/s41598-020-76744-5.
9. Redanz, S.; Cheng, X.; Giacaman, R.A.; Pfeifer, C.S.; Merritt, J.; Kreth, J. Live and let die: hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. *Mol. Oral Microbiol.* **2018**, *33*, 337-352.
10. Thein, Z.M.; Samaranayake, Y.H.; Samaranayake, L.P. Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. *Arch. Oral Biol.* **2006**, *51*, 672-680. doi: 10.1016/j.archoralbio.2006.02.005.
11. Romo, J.A.; Kumamoto, C.A. On commensalism of *Candida*. *J Fungi* (Basel) **2020**, *6*, 16. doi: 10.3390/jof6010016.
12. Simón-Soro, A.; Guillen-Navarro, M.; Mira, A. Metatranscriptomics reveals overall active bacterial composition in caries lesions. *J. Oral Microbiol.* **2014**, *6*, 25443. doi: 10.3402/jom.v6.25443.
13. Lozano, C.; Rodríguez, G.; Lefimil, C.; Morales-Bozo, I.; Urzúa-Orellana, B. Prevalence of *Candida albicans* and carriage of *Candida* non-*albicans* in the saliva of preschool children, according to their caries status. *Acta Odontol. Scand.* **2017**, *75*, 30–35. doi: 10.1080/00016357.2016.1244560.
14. Du, Q.; Ren, B.; He, J.; Peng, X.; Guo, Q.; Zheng, L.; Li, J.; Dai, H.; Chen, V.; Zhang, L.; Zhou, X; Xu, X. *Candida albicans* promotes tooth decay by inducing oral microbial dysbiosis. *ISME J.* **2021**, *15,* 894-908. doi: 10.1038/s41396-020-00823-8.
15. Díaz, P.I.; Strausbaugh, L.D.; Dongari-Bagtzoglou, A. Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. *Front. Cell. Infect. Microbiol.* **2014**, *4*, 101-106. doi: 10.3389/fcimb.2014.00101.
16. Janus, M.M.; Willems, H.M.E.; Krom, B.P. *Candida albicans* in multispecies oral communities; A keystone commensal? *Adv. Exp. Med. Biol.* **2016**, *931*, 13-20. doi: 10.1007/5584_2016_5.
17. Baraniya, D.; Chen, T.; Nahar, A.; Alakwaa, F.; Hill, J.; Tellez, M.; Ismail, A.; Puri, S.; Al-Hebshi, N.N. Supragingival mycobiome and inter-kingdom interactions in dental caries. *J. Oral Microbiol.* **2020**, *12*, 1729305-1729313. doi: 10.1080/20002297.2020.1729305.
18. Hong, B.Y.; Hoare, A.; Cardenas, A.; Dupuy, A.K.; Choquette, L.; Salner, A.L.; Schauer, P.K.; Hegde, U.; Petersen, D.E.; Dongari-Bagtzoglou, A.; Strausbauhg, L.D.; Díaz, P.I. The salivary mycobiome contains 2 ecologically distinct mycotypes. *J. Dent. Res.* **2020**, *99*, 730-738. doi: 10.1177/0022034520915879.
19. Li, W.; Yu, D.; Gao, S.; Lin, J.; Chen, Z.; Zhao, W. Role of *Candida albicans*-secreted aspartyl proteinases (Saps) in severe early childhood caries. *Int. J. Mol. Sci.* **2014**, *15*, 10766-10779. doi: 10.3390/ijms150610766.
20. de Abreu, T.C.; Barbosa, M.; Moraes, P.; Barbosa, G.F.; Cople, L.; Fonseca-Goncalves, A. Demineralizing potential of dental biofilm added with *Candida albicans* and *Candida parapsilosis* isolated from preschool children with and without caries. *Microb. Pathog.* **2016**, *100*, 51-55. doi: 10.1016/j.micpath.2016.09.003.
21. Lamont, R.J.; Jenkinson, H.F. Microbiology of Caries. In *Oral Microbiology at a Glance*, 1st ed.; Wiley-Blackwell publication, United Kingdom, 2010; Volume 1, pp. 34.
22. Akküç, S.; Duruk, G.; Keles, A. Remineralization effect of three different agents on initial caries and erosive lesions: a micro-computed tomography and scanning electron microscopy analysis. *BMC Oral Health* **2023**, *23*, 106-117. doi: 10.1186/s12903-023-02805-6.
23. Soon-Hwan, O.; Martin-Yken, H.; Coleman, D.A.; Dague, E.; Hoyer, L.L. Development and use of a monoclonal antibody specific for the *Candida albicans* cell-surface protein Hwp1. *Front. Cell. Infect. Microbiol.* **2022**, *12*, 907453. doi: 10.3389/fcimb.2022.907453.
24. Ten Cate, J.M.; Klis, F.M.; Pereira-Cenci, T.; Crielaard, W.; de Groot, P.W.J. Molecular and cellular mechanisms that lead to *Candida* biofilm formation. *J. Dent. Res.* **2009**, *88*, 105-115. doi: 10.1177/0022034508329273.

25. do Rosário Palma, A.; Domingues, N.; de Barros, P.; Brito, G.; Cardoso, A. Influence of *Streptococcus mitis* and *Streptococcus sanguinis* on virulence of *Candida albicans*: in vitro and in vivo studies. *Folia Microbiol.* **2019**, *64*, 215–222. doi: 10.1007/s12223-018-0645-9.
26. Brown, G. Innate antifungal immunity: the key role of phagocytes. *Ann. Rev. Immunol.* **2011**, *29*, 1-21. doi: 10.1146/annurev-immunol-030409-101229.
27. Nasution, O.; Srinivasa, K.; Kim, M.; Kim, Y.J.; Kim, W.; Jeong, W.; Choi, W. Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. *Eukaryot. Cell*. **2008**, *7*, 2008-2011. doi: 10.1128/EC.00105-08.
28. Koo, H.; Andes, D.R.; Krysan, D.J. Candida-streptococcal interactions in biofilm-associated oral diseases. *PLoS Pathog*. **2018**, *14*, e1007342. doi: 10.1371/journal.ppat.1007342.
29. Komalapriya, C.; Kaloriti, D.; Tillmann, A.T.; Yin, Z.; Herrero-de-Dios, C.; Jacobsen, M.D.; Belmonte, R.C.; Cameron, G.; Haynes, K.; Grebogi, C.; de Moura, A.P.; Gow, N.A.; Thiel, M.; Quinn, J.; Brown, A.J.; Romano, M.C. Integrative Model of Oxidative Stress Adaptation in the Fungal Pathogen *Candida albicans*. *PloS One* **2015**, *10*, e0137750. doi: 10.1371/journal.pone.0137750.
30. Díaz-Garrido, N.; Lozano, C.; Giacaman, R. Frequency of sucrose exposure on the cariogenicity of a biofilm-caries model. *Eur. J. Dent.* **2016**, *10*, 345–350. doi: 10.4103/1305-7456.184163.
31. Giacaman, R.A.; Umaña, R.; Nuñez, M.J.; Díaz-Garrido, N.; Echeverría, C.; García-Manríquez, N.; Mira, A.; Fernández, C.E.; Gambetta-Tessini, K.; Lozano C.P. Saliva decreases sucrose-induced cariogenicity in an experimental biological caries model. *Microorganisms* **2023**, *11*, 1426. doi: 10.3390/microorganisms11061426.
32. Lozano, C.; Díaz-Garrido, N.; Kreth, J.; Giacaman, R. *Streptococcus mutans* and *Streptococcus sanguinis* Expression of Competition-Related Genes, Under Sucrose. *Caries Res.* **2019**, *53*, 194–203. doi: 10.1159/000490950.
33. Bezerra, N.V.F.; Brito, A.C.M.; de Medeiros, M.M.D.; de França Leite, K.L.; Bezerra, I.M.; de Almeida, L.F.D.; Aires, C.P.; Cavalcanti, Y.W. Glucose supplementation effect on the acidogenicity, viability, and extracellular matrix of *Candida* single- and dual-species biofilms. *J. Investig. Clin. Dent.* **2019**, *10*, e12412. doi: 10.1111/jicd.12412.
34. Aitken-Saavedra, J.; Lund, R.G.; González, J.; Huenchunao, R.; Perez-Vallespir, I.; Morales-Bozo, I.; Urzúa, B.; Tarquinio, S.C.; Maturana-Ramírez, A.; Martos, J.; Fernandez-Ramires, R.; Molina-Berríos, A. Diversity, frequency and antifungal resistance of *Candida* species in patients with type 2 diabetes mellitus. *Acta Odontol. Scand*. **2018**, *76*, 580–586. doi: 10.1080/00016357.2018.1484154.
35. Domingues, P.C.A.; Oliveira, V.C.; Bim, F.L.; Aires, C.P.; Santos, A.P.D.; Castro, D.T.; Silva-Lovato, C.H.; Andrade, D.; Watanabe, E. Influence of glucose supplementation on biofilm formation of *Candida albicans* and *Candida glabrata* isolated from diabetic and non-diabetic individuals. *Arch. Oral Biol.* **2022**, *134*, 105339. doi: 10.1016/j.archoralbio.2021.105339.
36. Lok, B.; Ahmad, M.A.; Mohd, L.Z.; Chukwudi, N.A.; Sandai, R.; Sandai, D. The assimilation of different carbon sources in *Candida albicans*: Fitness and pathogenicity. *Med. Mycol.* **2021**, *59*, 115–125. doi: 10.1093/mmy/myaa080.
37. Xiang, Z.; Wakade, R.S.; Ribeiro, A.A.; Hu, W.; Bittinger, K.; Simon-Soro, A.; Kim, D.; Li, J.; Krysan, D. J.; Liu, Y.; Koo, H. Human Tooth as a Fungal Niche: *Candida albicans* Traits in Dental Plaque Isolates. *mBio*. **2023**, *14*, e0276922. doi: 10.1128/mbio.02769-22.
38. Ev, L.D.; Damé-Teixeira, N.; DO, T.; Maltz, M.; Cavalcanti, C. The role of *Candida albicans* in root caries biofilms: an RNA-seq analysis. *J. Appl. Oral Sci.* **2020**, *28*, e20190578. doi: 10.1590/1678-7757-2019-0578.
39. Ren, Z.; Jeckel, H.; Simon-Soro, A.; Xiang, Z.; Liu, Y.; Cavalcanti, I.M.; Xiao, J.; Tin, N-N.; Hara, A.; Drescher, K.; Koo, H. Interkingdom assemblages in human saliva display group-level surface mobility and disease-promoting emergent functions. *PNAS* **2022**, *119*, e2209699119. doi: 10.1073/pnas.2209699119.
40. Díaz-Garrido, N.; Lozano, C.P.; Kreth, J.; Giacaman, R.A. Extended biofilm formation time by *Streptococcus sanguinis* modifies its non-cariogenic behavior, *in vitro*. *Braz. Oral Res.* **2022**, *36*, e107. 10.1590/1807-3107bor-2022.vol36.0107.
41. Chen, H.; Zhou, X.; Ren, B.; Cheng, L. The regulation of hyphae growth in *Candida albicans*. *Virulence* **2020**, *11*, 337–348. doi: 10.1080/21505594.2020.1748930.
42. Lohse, M.B.; Gulati, M.; Johnson, A.D.; Nobile, C.J. Development and regulation of single- and multi-species *Candida albicans* biofilms. *Nat. Rev. Microbiol.* **2018**, *16*, 19–31. doi: 10.1038/nrmicro.2017.107.
43. Morse, D.J.; Wilson, M.J.; Wei, X.; Bradshaw, D.J.; Lewis, M.A.O.; Williams, D.W. Modulation of *Candida albicans* virulence in in vitro biofilms by oral bacteria. *Lett. Appl. Microbiol*. **2019**, *68*, 337–343. doi: 10.1111/lam.13145.
44. Xu, H.; Sobue, T.; Bertolini, M.; Thompson, A.; Vickerman, M.; Nobile, C.J.; Dongari-Bagtzoglou, A.D. *S. oralis* activates the Efg1 filamentation pathway in *C. albicans* to promote cross-kingdom interactions and mucosal biofilms. *Virulence* **2017**, *8*, 1602–1617. doi: 10.1080/21505594.2017.1326438.
45. Basso, V.; d'Enfert, C.; Znaidi, S.; Bachellier-Bassi, S. From Genes to Networks: The Regulatory Circuitry Controlling *Candida albicans* Morphogenesis. *Curr. Top. Microbiol. Immunol.* **2019**, *422*, 61–99. doi: 10.1007/82_2018_144.

46. Merino Guzmán, G.; Cedillo Ramírez, L.; Silva Andrade, F.; Muñoz García, A.A.; Castañeda Roldán, E.I. Análisis morfológico de biopelículas de *Candida albicans* producidas en diferentes condiciones de pH y temperatura analizadas por microscopía óptica y de fuerza atómica. [In English: Morphological analysis of *Candida albicans* biofilms produced at different pH and temperature conditions analyzed by optic and atomic force microscopy]. *Rev. Mex. Mic*. **2011**, *33*, 1-8.
47. Westwater, C.; Balish, E.; Schofield, D.A. *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. *Eukaryot. Cell*. **2005**, *4*, 1654–1661. doi: 10.1128/EC.4.10.1654-1661.2005.
48. Willems, H.M.; Kos, K.; Jabra-Rizk, M. A.; Krom, B.P. *Candida albicans* in oral biofilms could prevent caries. *Pathog. Dis.* **2016**, *74*, ftw039. doi: 10.1093/femspd/ftw039.
49. Eidt, G.; Gomes de Andrade, C.; de Cássia, T.; Arthur, R.A. Role of *Candida albicans* on enamel demineralization and on acidogenic potential of Streptococcus mutans in vitro biofilms. *J. Appl. Oral Sci.* **2019**, *27*, e20180593. doi: 10.1590/1678-7757-2018-0593.
50. Huang, X.; Schulte, R.M.; Burne, R.A.; Nascimento, M.M. Characterization of the arginolytic microflora provides insights into pH homeostasis in human oral biofilms. *Caries Res*. **2015**, *49*, 165-176. doi:10.1159/000365296.
51. Díaz-Garrido, N.; Lozano, C.P.; Kreth, J.; Giacaman, R.A. Competition and caries on enamel of a dual-species biofilm model with *Streptococcus mutans* and *Streptococcus sanguinis*. *Appl. Environ. Microbiol.* **2020**, *86*, e01262-20. doi: 10.1128/AEM.01262-20.
52. Szabó; B.; Majoros, L.; Papp-Falusi, E.; Szabó, Z.; Szabó, J.; Márton, I.; Kelentey, B. Studies on the possible aetiological role of different *Candida* species in pathogenesis of dentine caries by monitoring the calcium release from tooth particles. *Acta Microbiol. Immunol. Hung*. **2014**, *61*, 11-17. doi: 10.1556/AMicr.61.2014.1.2.
53. Charone, S.; Portela, M.B.; Martins, K.O.; Soares, R.M.; Castro, G.F. Role of *Candida* species from HIV infected children in enamel caries lesions: an in vitro study. *J. Appl. Oral Sci.* **2017**, *25*, 53–60. doi: 10.1590/1678-77572016-0021.
54. Kreth, J.; Zhang, Y.; Herzberg, M. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *J. Bacteriol.* **2008**, *190*, 4632–4640. doi: 10.1128/JB.00276-08.
55. Zheng, L.; Chen, Z.; Itzek, A.; Herzberg, M.C.; Kreth, J. CcpA regulates biofilm formation and competence in *Streptococcus gordonii*. *Mol. Oral Microbiol.* **2012**, *27*, 83–94. doi: 10.1111/j.2041-1014.2011.00633.x.
56. d'Enfert, C.; Kaune, AK.; Alaban, LR.; Chakraborty, S.; Cole, N.; Delavy, M.; et al. The impact of the Fungus-Host-Microbiota interplay upon *Candida albicans* infections: current knowledge and new perspectives. *FEMS Microbiol. Rev.* **2021**, *45*, fuaa060. doi: 10.1093/femsre/fuaa060.
57. Vila, T.; Kong, E.F.; Ibrahim, A.; Piepenbrink, K.; Shetty, A.C.; McCracken, C.; Bruno, V.; Jabra-Rizk, M.A. *Candida albicans* quorum-sensing molecule farnesol modulates staphyloxanthin production and activates the thiol-based oxidative-stress response in *Staphylococcus aureus*. *Virulence* **2019**, *10*, 625–642. doi: 10.1080/21505594.2019.1635418.
58. Sebaa, S.; Boucherit-Otmani, Z.; Courtois, P. Effects of tyrosol and farnesol on *Candida albicans* biofilm. *Mol. Med. Rep.* **2019**, *19*, 3201–3209. doi: 10.3892/mmr.2019.9981.
59. Bowen, W.; Burne, R.; Wu, H.; Koo, H. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. *Trends Microbiol.* **2018**, *26*, 229–242. doi: 10.1016/j.tim.2017.09.008.