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ORIGINAL ARTICLE

Influence of sucrose on growth and sensitivity of *Candida albicans* alone and in combination with *Enterococcus faecalis* and *Streptococcus mutans* to photodynamic therapy

Fernanda Malagutti Tomé[1] · Lucas De Paula Ramos[1] · Fernanda Freire[1] · Cristiane Aparecida Pereira[1] · Ingrid Christine Barbosa de Oliveira[1] · Juliana Campos Junqueira[1] · Antonio Olavo Cardoso Jorge[1] · Luciane Dias de Oliveira[1]

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Abstract This study has evaluated the effects of photodynamic inactivation (PDI) using erythrosine as photosensitizer and green light-emitting diode (LED) on biofilms of *Candida albicans* alone and in combination with *Enterococcus faecalis* and *Streptococcus mutans*. We have also evaluated the effect of sucrose on biofilm formation and bacterial growth and sensitivity to PDI. Biofilms were formed in suspension of 10^6 cells/ml on plates before being grown in broth culture with and without sucrose and incubated for 48 h. Next, the treatment was applied using erythrosine at a concentration of 400 μM for 5 min and green LED (532 ± 10 nm) for 3 min on biofilms alone and in combination. The plates were washed and sonicated to disperse the biofilms, and serial dilutions were carried and aliquots seeded in Sabouraud agar before incubation for 48 h. Next, the colony-forming units per milliliter (CFU/ml; $\log_{10}$) were counted and analyzed statistically (ANOVA, Tukey test, $P \leq 0.05$). Results show that *S. mutans* favors the growth of *C. albicans* in biofilms with sucrose, with treatment not being effective. However, when the biofilm was grown without sucrose, we found a reduction in biofilm formation and a significant decrease in the PDI treatment ($P < 0.0001$). In conclusion, both growth and sensitivity to PDI in biofilms of *C. albicans* are strongly influenced by bacterial combination, and the presence of sucrose affected directly the growth and sensitivity of the biofilm to PDI as sucrose is the substrate for construction of the exopolysaccharide matrix.

Keywords Biofilm · Sucrose · Photodynamic therapy · *Enterococcus faecalis* · *Streptococcus mutans* · *Candida albicans*

✉ Fernanda Malagutti Tomé
fmtome@gmail.com

Lucas De Paula Ramos
lucas93paula@hotmail.com.br

Fernanda Freire
fefreire21@hotmail.com

Cristiane Aparecida Pereira
cristiane.pereira@fosjc.unesp.br

Ingrid Christine Barbosa de Oliveira
ingrid.oliveira@fosjc.unesp.br

Juliana Campos Junqueira
juliana@fosjc.unesp.br

Antonio Olavo Cardoso Jorge
olavojorge@fosjc.unesp.br

Luciane Dias de Oliveira
luciane@fosjc.unesp.br

[1] Department of Biosciences and Oral Diagnosis, São Paulo State University (Unesp), Institute of Science and Technology, São José dos Campos, Francisco José Longo 777, São Dimas, São José dos Campos, SP 12245-000, Brazil

Introduction

Biofilms develop after primary colonizing microorganisms adhere to inert or chemically conditioned surfaces. During biofilm maturation, new microorganism species establish as secondary colonizers on the top of the earlier layers. The structure and integrity of a biofilm is secured by the production of macromolecular extracellular substances. Depending on the species composition, there is intense inter- and intra-species communication in polymicrobial oral biofilms [1, 2]. Oral microbial biofilms may contain several hundred species,

and their composition can differ significantly between individuals [3, 4].

Because of the importance of the microbial interactions, several studies have been conducted with mixed biofilms. Cruz et al. [5] further investigated how *Enterococcus faecalis* and *Candida albicans* affect each other in the context of an infection. Shekh and Roy [6] reported the purification and characterization of an antifungal protein produced by *E. faecalis* that shows broad-spectrum activity against the indicator organism, multidrug-resistant *C. albicans*, with negligible hemolytic activity. Brusca et al. [7] used mathematical modeling to study the potential interaction between *C. albicans* and *Streptococcus mutans* and to describe the mechanisms of interaction between both microorganisms.

Microorganisms are found in the nature forming biofilms, which are organized structured communities enclosed within a matrix of extracellular material. Biofilm cells are phenotypically different from planktonic cells as the structure of the former confers resistance to antifungal agents, which is related to the limited diffusion of the agent through the extracellular polysaccharide matrix or to phenotypic and genotypic alterations [8].

In view of the increase in opportunistic infections caused by species of the genus *Candida* in immune-suppressed patients, and the consequent emergence of strains resistant to conventional antifungal agents, new treatment options for oral candidoses are required to improve the therapeutic arsenal. In this respect, photodynamic inactivation (PDI) has been proposed as a new option to reduce yeasts of the genus *Candida* in the oral cavity [8–12].

PDI is based on the concept that a non-toxic dye, known as a photosensitizer, may be located preferentially in certain tissues or cells and subsequently activated by harmless visible light to produce reactive oxygen species which can kill cells binding to the photosensitizer. Numerous published studies have demonstrated that PDI is highly effective in the inactivation of fungi in vitro [13–16]. Additionally, it is believed that the development of resistance by microorganisms to PDI is an unlikely event as this is considered a typical multitarget process, which is different compared to most antifungal agents [17].

Clearly, the structure of the polysaccharide matrix can play a critical role in the virulence of plaque biofilms by influencing their physical and biochemical properties. It can enhance adherence of microorganisms, promote coherence, act as reserve source of energy, protect microorganisms from inimical influences, and affect the diffusion of substances into and out of the biofilm. In fact, sucrose is the raw material for the construction of the polysaccharide matrix [18–20].

Based on the above findings, the presence of mixed biofilm and influence of sucrose on the development and resistance of bacteria seem to affect their response to photodynamic therapy. This is the first study to report the action of PDI considering the interactions of microorganisms and biofilm growth with sucrose and without sucrose. The objective of this study was to analyze the sensitivity of *C. albicans* alone and in association with *E. faecalis* and *S. mutans* with and without sucrose to photodynamic therapy using erythrosine dye and green light-emitting diode (LED).

Materials and methods

Preparation of the microorganism suspensions

Three reference strains (American Type Culture Collection, ATCC), namely, *C. albicans* (ATCC 18804), *E. faecalis* (ATCC 29212), and *S. mutans* (ATCC 35688), were used in the study.

C. albicans was plated onto Sabouraud dextrose agar (HiMedia, Mumbai, India) and *S. mutans* and *E. faecalis* onto brain heart infusion (BHI) agar (Acumedia, Neogen, Lasing, MI, USA) incubated at 37 °C for 24 h to reactivate the frozen strains. All experiments with *S. mutans* were incubated at 37 °C and 5% CO_2.

After incubation, the culture seeded in agar was suspended in sterile BHI broth (Acumedia, Neogen, Lasing, MI, USA) with or without 5% of sucrose. This suspension was standardized using a spectrophotometer (B582, Micronal, São Paulo, SP, Brazil) adjusting the density of suspension equivalent to 10^6 cells/ml. The optical density and wavelength parameters used were, respectively, 0.284 and 530 nm for *C. albicans*, 0.620 and 398 nm for *S. mutans*, and 0.298 and 398 nm for *E. faecalis*.

Biofilm formation

After standardization to grow the biofilms, the suspensions of each microorganism were distributed into 96 wells of the microtiter plate with high-transparency polystyrene and smooth and polished well walls, sterilized by gamma ray (Costar Corning, New York, USA), in a total volume of 250 μl, which was divided into equal proportions of each microorganism in mixed biofilms. We emphasized the presence or absence of sucrose in the broth suspension, thus creating two evaluation biases. The groups and volumes were accordingly seeded as shown in Table 1. The plates were incubated for 24 h at 37 °C in a microaerophilic atmosphere by using a 3D shaker (GyroTwister™ 3-D Shaker, Labnet, NJ, USA).

Photosensitizer and LED

For sensitization of biofilms, erythrosine (ER) was used as photosensitizer (PS) (Sigma-Aldrich) at absorption wavelengths of 500–550 nm and concentration of 400 μM. The PS solution was prepared by dissolving the powdered dye in

Table 1 Distribution of experimental groups

Groups		Ca	Ca + Sm	Ca + Ef	Ca + Ef + Sm
Volumes		250 μl	125 + 125 μl	125 + 125 μl	83.3 + 83.3 + 83.3 μl
I	Control group (P−L−)	$N = 10$	$N = 10$	$N = 10$	$N = 10$
II	Photosensitizer group (P+L−)	$N = 10$	$N = 10$	$N = 10$	$N = 10$
III	Light group (P−L+)	$N = 10$	$N = 10$	$N = 10$	$N = 10$
IV	PDI group (P+L+)	$N = 10$	$N = 10$	$N = 10$	$N = 10$

Volumes: values used for each standardized suspension, in each experimental group in the biofilm assembly

Ca Candida albicans isolated, *Ca + Sm C. albicans* combined with *Streptococcus mutans*, *Ca + Ef C. albicans* combined with *E. faecalis*, *Ca + Ef + Sm C. albicans* combined with *E. faecalis* and *S. mutans*

PBS, filtered through a sterile 0.22-μm filter (Millipore, São Paulo, Brazil), and stored in the dark before use.

A green light-emitting diode (LED) (MMOptics, São Carlos, SP, Brazil) was used as light source operating at wavelengths of 532 ± 10 nm (corresponding to the maximum absorption of ER), output power of 90 mW, energy of 5.4 J, fluence rate of 237 mW/cm^2, and fluence of 14 J/cm^2 for 90 s. The irradiated area of the biofilm was 0.38 cm^2. The temperature at the bottom of the 96-well microtiter plates was monitored by using an infrared thermometer (MX4, Raytek, Sorocaba, SP, Brazil), with no increase being observed in the temperature after LED irradiation.

Treatment

The biofilms were submitted to different experimental conditions, namely, I—control group (P−L−), using 250 μl of sterile saline solution [0.9% sodium chloride (NaCl)]; II—photosensitizer group (P+L−), using sensitization with 250 μl of ER at a concentration of 400 μM for 5 min; III—light group (P−L+), using 250 μl of 0.9% NaCl and LED irradiation for 90 s; and IV—PDI group (P+L+) using sensitization with 250 μl of ER at a concentration of 400 μM for 5 min and LED irradiation for 90 s.

The plates with photosensitizer solution were shaken for 5 min (pre-irradiation time) in an orbital shaker (Solab, Piracicaba, Brazil). The irradiation of biofilms was performed in the dark under aseptic conditions in a laminar flow hood. During irradiation, the plates were covered with a black matte screen with an orifice of the same size as the wells to prevent light from reaching the neighboring wells.

Determination of colony-forming units per milliliter

After irradiation of the biofilms, the photosensitizer solution was washed out with 250 μl 0.9% NaCl. Next, the biofilms were disaggregated in 0.9% NaCl with ultrasonic sonicator (Sonics Vibra-Cell, CT, USA) for 30 s. Biofilm suspensions were serially diluted in 0.9% NaCl to produce dilutions of 10^{-1} to 10^{-4} times the original concentration. Aliquots of 0.1 ml of each dilution were seeded on agar plates according to each microorganism of the biofilm. Sabouraud dextrose agar (HiMedia) with 50 mg/l chloramphenicol (União Química, São Paulo, Brazil) was used for *C. albicans*, Mitis Salivarius Agar (Difco, Detroit, USA) supplemented with 0.2 IU/ml bacitracin (União Química, São Paulo, Brazil) and 15% sucrose was used for *S. mutans*, and *m-Enterococcus* agar (Difco, Detroit, USA) was used for *E. faecalis*. The number of colony-forming units per milliliter (CFU/ml) was determined after 48 h of incubation. The results were log-transformed ($\log_{10}$) and analyzed by using ANOVA and Tukey's test, with a P value less than 5% indicating statistically significant difference. To validate the mixed biofilms, the counting of *C. albicans*, *E. faecalis*, and *S. mutans* was performed separately.

Influence of sucrose on biofilm and effects of PDI

The previously described procedures were repeated by using BHI broth (Acumedia) without the addition of sucrose in order to verify the influence of sucrose on the production of the exopolysaccharide matrix by *S. mutans*, and consequently its influence on both biofilm and effects of PDI.

Statistical analysis

The results were log-transformed ($\log_{10}$) and analyzed by using analysis of variance (ANOVA) and Tukey's test. Statistically significant difference was set at $p < 0.05$.

Results

This study was divided into two parts, biofilms grown with and without sucrose submitted to different experimental conditions of PDI assays. Mean and standard deviation values of CFU/ml ($\log_{10}$) obtained under different experimental conditions for each biofilm group are shown in Figs. 1, 2, 3, and 4. The mean values of reduction (CFU $\log_{10}$) and P values obtained for growth interactions are shown in Table 2. Table 3

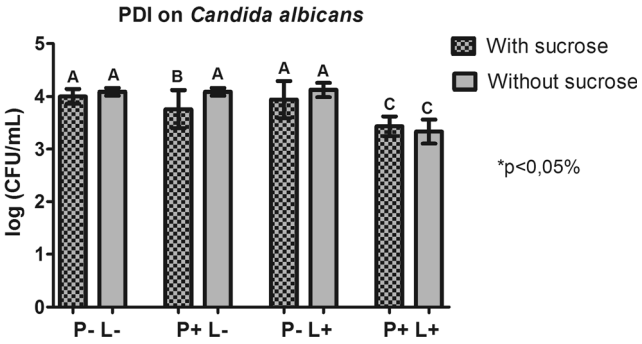

Fig. 1 Mean ($n = 10$) and standard deviation values of CFU/ml ($\log_{10}$) of biofilms formed by *C. albicans* (C) with and without sucrose under the following different experimental conditions: control (P−L−, $n = 10$), with 250 μl of 0.9% NaCl; (P+L−, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min; (P−L+, $n = 10$), treated with 250 μl of 0.9% NaCl and LED irradiation for 90 s; and (P+L+, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min and LED irradiation 90 s. Tukey's test was used for each group tested. Values followed by different *capital letters* differed significantly between the experimental conditions ($P < 0.05$)

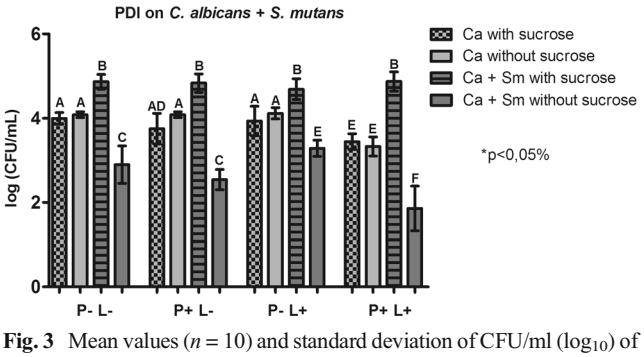

Fig. 3 Mean values ($n = 10$) and standard deviation of CFU/ml ($\log_{10}$) of biofilms formed by *C. albicans* (C) compared with *C. albicans* + *S. mutans* (CM) association both with and without sucrose for different experimental conditions: control (P−L−, $n = 10$), treated with 250 μl of 0.9% NaCl; (P+L−, $n = 10$): sensitization with 250 μl of ER at a concentration of 400 μM for 5 min; (P−L+, $n = 10$): treated with 250 μl of 0.9% NaCl and LED irradiation for 90 s; (P+L+, $n = 10$), sensitization with 250 μl of ER at a concentration of 400 μM for 5 min and LED irradiation for 90 s. Tukey's test was used for each group tested. Values followed by different *capital letters* differed significantly among the experimental conditions ($P < 0.05$)

shows the mean values of reduction (CFU $\log_{10}$) and P values for biofilms submitted to PDI mediated by ER (P+L+) compared to untreated control biofilms (P−L−).

As shown in Fig. 1, no statistical difference was found in the growth of *C. albicans* biofilms with or without sucrose. The efficiency of PDI for *C. albicans* biofilms was statistically significant under both conditions, with P values =0.0200 with sucrose and <0.0001 without sucrose (Table 2). Thus, one can see that sucrose had no influence on the growth of *C. albicans* biofilm.

The combination of *C. albicans* with *E. faecalis* had lower growth in the biofilm (Fig. 2). But the reduction of biofilm in the PDI group was not significant with the presence of sucrose ($P = 0.2301$), showing a significant reduction without it ($P = 0.0002$).

When *C. albicans* grown in combination with *S. mutans* (Fig. 3) in the presence of sucrose showed an increase in the biofilm growth, the treatment with PDI did not show efficiency. On the other hand, in the same biofilm grown without sucrose, there was a significant reduction in the biofilm treated with PDI ($P = <0.0001$).

One can observe multiple species in the biofilm formed with *C. albicans*, *E. faecalis*, and *S. mutans* (Fig. 4), with the addition of sucrose increasing the growth of the yeast, but with no reduction of PDI assays. For biofilms formed without sucrose, one can see that there was a reduction in

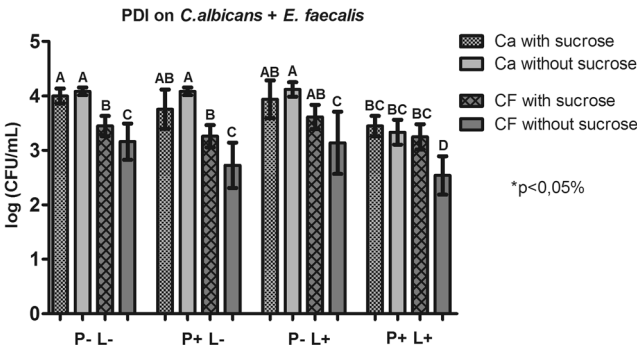

Fig. 2 Mean ($n = 10$) and standard deviation values of CFU/ml ($\log_{10}$) of biofilms formed by *C. albicans* (C) compared to the *C. albicans* + *E. faecalis* combination with and without sucrose under the following different experimental conditions: control (P−L−, $n = 10$), treated with 250 μl of 0.9% NaCl; (P+L−, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min; (P−L+, $n = 10$), treated with 250 μl of 0.9% NaCl and LED irradiation for 90 s; and (P+L+, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min and LED irradiation for 90 s. Tukey's test was used for each group tested. Values followed by different *capital letters* differed significantly between the experimental conditions ($P < 0.05$)

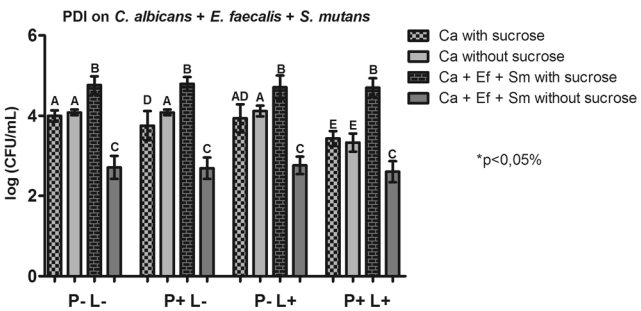

Fig. 4 Mean ($n = 10$) and standard deviation values of CFU/ml ($\log_{10}$) of biofilms formed by *C. albicans* (C) compared to the *C. albicans* + *S. mutans* + *E. faecalis* (CMF) combination with and without sucrose under the following different experimental conditions: control (P−L−, $n = 10$), treated with 250 μl of 0.9% NaCl; (P+L−, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min; (P−L+, $n = 10$), treated with 250 μl of 0.9% NaCl and LED irradiation for 90 s; and (P+L+, $n = 10$), sensitization with 250 μl of ER at concentration of 400 μM for 5 min and LED irradiation for 90 s. Tukey's test was used for each group tested. Values followed by different *capital letters* differed significantly between the experimental conditions ($P < 0.05$)

Table 2 Log_{10} results analyzed by *t* test obtained for control biofilms, considering the growth of *Candida albicans* biofilm (C) alone versus combination with *Enterococcus faecalis* (CF), *Streptococcus mutans* (CM), and *Streptococcus mutans* and *Enterococcus faecalis* (CMF), with and without sucrose

Groups	With sucrose Log_{10}	*P value	Log_{10} Without sucrose	*P value
C × CM	0.867	<0.0001	1.19	<0.0001
C × CF	0.552	<0.0001	0.927	<0.0001
C × CMF	0.763	<0.0001	1.38	<0.0001

*$P > 0.05$, statistically significant difference (ANOVA, Tukey's test)

the growth, but with no statistically significant reduction in the group treated with PDI.

Table 2 shows that the amount of *C. albicans* biofilm combined with *S. mutans* (0.876 log_{10}, $P < 0.0001$) or of *C. albicans* with *E. faecalis* and *S. mutans* (0.763 log_{10}, $P < 0.0001$) in the presence of sucrose was greater than that of the biofilm formed by one yeast alone. However, when combined with *E. faecalis*, the growth was reduced (0.552 log_{10}) ($P < 0.0001$). In the absence of sucrose in the biofilm of *C. albicans* with *S. mutans* (1.19 log_{10}, $P < 0.0001$), there was a lower growth compared to biofilm of *C. albicans* alone. This decrease was also observed in biofilms presenting combination of *C. albicans* with *E. faecalis* and *S. mutans* (1.38 log_{10}, $P < 0.0001$) and in biofilms *of C. albicans* combined with *E. faecalis* (0927 log_{10}, $P < 0.0001$).

As shown in Table 3, the biofilms formed in the presence or absence of sucrose showed different sensitivities to PDI. In the presence of sucrose, only biofilms of *C. albicans* alone showed sensitivity to PDI, with a significant reduction (log_{10} 0.5518, $P = 0.0200$). However, in groups without sucrose, only the combination of *C. albicans*, *S. mutans*, and *E. faecalis* (CMF) showed no statistically significant reduction (0.1080 log_{10}) ($P = 0.8787$).

Table 3 Log_{10} results of reduction analyzed by *t* test obtained for biofilms submitted to PDT mediated by erythrosine dye (F+L+) in relation to untreated control biofilms (F−L−), considering *Candida albicans* (C) alone and combined with *Enterococcus faecalis* (CF) and *Streptococcus mutans* (CM), with and without sucrose

Groups	With sucrose Log_{10} reduction	*P value	Without sucrose Log_{10} reduction	*P value
C	0.5518	0.02	0.754	<0.0001
CF	0.2005	0.2301	0.619	0.0002
CM	0.0051	>0.9999	1.039	<0.0001
CMF	0.0581	0.9457	0.108	0.8787

*$P > 0.05$, statistically significant difference (ANOVA, Tukey's test)

Discussion

Biofilms formed by only *C. albicans* were sensitive to PDI mediated by ER as photosensitizer and green LED, both with and without sucrose. PDI significantly reduced the amounts of yeast by 0.5518 log_{10} ($P = 0.0200$) and 0.7540 log_{10} ($P \leq 0.0001$), respectively, with and without sucrose. These results are corroborated by other similar studies. Costa et al. [21] evaluated the effect of PDI with ER and green LED on planktonic cultures and biofilms of *C. albicans*, reporting that biofilms were more resistant than the planktonic forms. Another study by Costa et al. [22] investigated the effects of PDI on *C. albicans* planktonic cultures and biofilms by using either Rose Bengal (RB) dye or ER with green LED, reporting that PDI was effective in reducing *C. albicans*.

Costa et al. [23] evaluated the effects of PDI on oral candidiasis in mice and in vitro regarding the adherence of yeast to oral epithelial cells (OECs), which were submitted to PDI with erythrosine (400 μmol/l) and green LED at 14.34 J/cm^{-2}. PDI reduced significantly the amount of yeast present in the lesions by (0.73 log_{10}) ($P = 0.018$) and the *C. albicans* adherence to OECs by 35% without damaging adjacent tissues ($P = 0.045$). The conclusion of this study was that PDI exhibited antifungal effects on *C. albicans* biofilms formed in vivo and reduced the capacity of *C. albicans* to adhere to OECs in vitro. A reduction of *C. albicans* after treatment with PDI was also observed.

The yeast *C. albicans* and the Gram-positive bacterium *E. faecalis* are both normal residents of the human oral microbiome, causing opportunistic disseminated infections in immunocompromised individuals. According to Garsin and Lorenz [24], who used a nematode infection model, that co-infection resulted in less pathology and less mortality than the infection with either species alone. This finding was partly explained by an inter-kingdom signaling event in which a bacterial-derived product inhibits hyphal morphogenesis of *C. albicans*.

In this study, when *C. albicans* was combined with *E. faecalis*, the yeast showed lower growth with and without sucrose. Also, *C. albicans* was not sensitive to PDI in the group with sucrose (reduction of 0.2005 log_{10}) ($P = 0.2301$), but was in the group without sucrose (reduction of 0.6190 log_{10}) ($P = 0.0002$). In a research study conducted by Shekh and Roy [6], an anti-*Candida* factor produced by *E. faecalis* (protein ACP) was purified and partially characterized. The ACP showed broad-spectrum activity against different multidrug-resistant strains of *C. albicans*. This finding helps explain the results of our study because the growth of *C. albicans* was reduced in the presence of *E. faecalis*. However, it is not clear why the yeast did not show significant reduction following PDI.

When *C. albicans* was combined with *S. mutans* with sucrose, it was found that growth increased (0.867 $\log_{10}$) ($P > 0.0001$) and the yeast was not sensitive to PDI (0.0051 $\log_{10}$) ($P > 0.9999$). This seems to increase the production of the extracellular polysaccharide (EPS) matrix by *S. mutans*, since we observed that the group without sucrose showed lower growth (1.19 $\log_{10}$) ($P < 0.0001$) and was sensitive to PDI (1.03 $\log_{10}$) ($P > 0.0001$). These data demonstrate that the more complex the composition of the biofilms, the more resistant they seem to be to the PDI process. The interactions between different matrix polymers produced by different microorganisms might result in a more viscous matrix [18, 20].

Clearly, the structure of the polysaccharide matrix can play a critical role in the virulence of plaque biofilms by influencing their physical and biochemical properties. It can enhance adherence of microorganisms, promote coherence, act as reserve source of energy, protect microorganisms from inimical influences, and affect diffusion of substances into and out of the biofilm [18]. Thus, it is likely that this matrix affects the absorption of erythrosine in the biofilm and the light penetration as well, thereby preventing the photodynamic action.

According to Falsetta et al. [25], *C. albicans* cells are frequently detected along with heavy infection by *S. mutans* in biofilms. The authors showed that the ability of these organisms to form biofilms together is enhanced in vitro and in vivo. The presence of *C. albicans* increases the production of EPS so that co-species biofilms accumulate more biomass and harbor more viable *S. mutans* cells than single-species biofilms.

The resulting three-dimensional biofilm architecture displays sizeable *S. mutans* microcolonies surrounded by fungal cells, which are enmeshed in a dense EPS-rich matrix. According to Sztajer et al. [26], in vitro data also revealed that glucosyltransferase-derived EPS is a key mediator of co-species biofilm development and that co-existence with *C. albicans* induces the expression of virulence genes in *S. mutans*. In this study, it was also found that *Candida*-derived β1,3-glucans contribute to the EPS matrix structure, whereas fungal mannan and β-glucan provide sites for binding and activity of glucosyltransferases. Altogether, they demonstrate a novel mutualistic bacterium-fungus relationship occurring at a clinically relevant site to amplify the severity of a ubiquitous infectious disease. [25].

The results of this study allow us to conclude that both growth and sensitivity of *C. albicans* biofilm to PDI are strongly influenced by the combination of this yeast with *S. mutans* in the biofilm. The presence of sucrose directly affects the growth and sensitivity of the biofilm as sucrose is the substrate for the synthesis of the EPS matrix.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Funding The authors declare that they did not receive funding for the development of this research.

Ethical procedures All ethical procedures were followed. Because the research used ATCC strains of microorganisms and did not involve any kinds of animal, the approval committee of ethics and informed consent do not apply to work.

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