

In Vitro Effect of Low-Level Laser Therapy on Typical Oral Microbial Biofilms

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The aim of this study was to evaluate the effect of specific parameters of low-level laser therapy (LLLT) on biofilms formed by *Streptococcus mutans*, *Candida albicans* or an association of both species. Single and dual-species biofilms - SSB and DSB - were exposed to laser doses of 5, 10 or 20 J/cm² from a near infrared InGaAsP diode laser prototype (LASERTable; 780 ± 3 nm, 0.04 W). After irradiation, the analysis of biofilm viability (MTT assay), biofilm growth (cfu/mL) and cell morphology (SEM) showed that LLLT reduced cell viability as well as the growth of biofilms. The response of *S. mutans* (SSB) to irradiation was similar for all laser doses and the biofilm growth was dose dependent. However, when associated with *C. albicans* (DSB), *S. mutans* was resistant to LLLT. For *C. albicans*, the association with *S. mutans* (DSB) caused a significant decrease in biofilm growth in a dose-dependent fashion. The morphology of the microorganisms in the SSB was not altered by LLLT, while the association of microbial species (DSB) promoted a reduction in the formation of *C. albicans* hyphae. LLLT had an inhibitory effect on the microorganisms, and this capacity can be altered according to the interactions between different microbial species.

Key Words: Biofilm, *Candida albicans*, low level laser therapy, *Streptococcus mutans*.

INTRODUCTION

The action of low-level laser therapy (LLLT) seems to be related to biostimulatory events (1). It is believed to accelerate wound healing capacity by increasing the synthesis of collagen and other proteins as well as the proliferation of cells involved in these events. Decrease of inflammatory response and analgesia are other effects of low-power lasers (1,2). LLLT has also been shown to have a significant bactericidal potential without causing damage to the oral tissues (3). This effect has been documented both *in vitro*, on diverse microbial species, and *in vivo*, in the treatment of gingivitis, periodontitis and other oral diseases (3-6).

It has been shown that LLLT associated or not with a photosensitizer can cause destruction of oral bacterial and fungal species (7). In these cases, the antibacterial mechanism of action of LLLT seems to be related to thermal effects and photo-disruption (7). However, LLLT may not cause immediate cell death, but it causes sublethal damages, such as destruction of the bacterial cell wall and accumulation of denatured protein in the bacterial cytoplasm (8). The integrity of the bacterial cell wall is closely related to the stability of gram-positive bacteria, such as *Streptococcus mutans*. Thus, damage to the bacterial cell wall may lead to growth inhibition and consequent cell lysis (9). Cytoplasmic accumulation of denatured proteins may induce cellular stress in an

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attempt to maintain homeostasis, causing the death of the microorganism (8).

LLLT may also cause disruption of microorganisms by altering the interaction among themselves and with the substrate (10). However, the literature shows that the effects of irradiation on bacterial biofilms seem to be species-dependent (6).

Although oral microorganisms can coexist in an independent manner, they frequently interact, forming communities, which have been referred to as biofilms (11). The microcolonies that form the biofilm may be composed of populations of one or more microbial species, depending on the environmental conditions in which they are formed, such as properties of the surface to which they adhere, presence of nutrients, pH and oxygen availability (12). In some cases, the interaction of different microbial species, such as bacteria and fungi, in biofilm formation, may promote relationships of cooperation among species, such as commensalism and mutualism, increasing their chances of survival and growth (11). The association between *S. mutans* and *Candida albicans* is one of the most frequently studied interactions of microbial species (12). *S. mutans* is one of the main bacterial strains colonizing the oral cavity (13). *C. albicans* is a commensal yeast of the oral cavity, which, under specific conditions like immunosuppression, may become pathogenic, causing candidiasis and affecting life quality by increasing patient morbidity (13).

It has been described that the interaction between microorganisms of different species may alter their behavior and growth (14). This study evaluated the effect of LLLT with specific parameters on single-species biofilms (SSB) of *S. mutans* and *C. albicans* and dual-species biofilms (DSB) associating both microorganisms.

MATERIAL AND METHODS

Microorganisms

Reference strains of *S. mutans* (UR 158) and *C. albicans* (ATCC 90028) were used for the formation of SSB and DSB.

The microorganisms were grown in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) at 37°C during 18 h according to their physiological demands. These cultures were centrifuged at 1,500 xg for 10 min and washed with sterile phosphate buffer saline (PBS - pH 7.4). The optical density (O.D.) of these

suspensions were determined at 660 nm (Eppendorf AG 22331; Eppendorf, Hamburg, Germany) and adjusted to values of 0.15 and 1.0 for *S. mutans* and *C. albicans*, respectively, which correspond to a stock suspension of 10⁷ colony-forming units *per* milliliter (cfu/mL).

Microbial Biofilms

Biofilm growth on cell culture plates was done as described by Thein et al. (15). For SSB formation, 1 mL of the suspension was transferred to each well of a culture plate. For DSB formation, equal volumes of suspension of each monoculture were mixed to reach a final volume of 1 mL and were transferred to the wells of a culture plate (1 mL/well). Suspensions of each microbial strain served as controls. The plates were incubated at 37°C during 1 h and 30 min in an orbital agitator at 75 rpm to promote adhesion of the microorganisms to the surfaces of the wells. After the adhesion period, the cell suspension was aspirated and each well was washed twice with sterile PBS for removal of non-adhered microorganisms. Then, 1 mL of BHI was added to the each well to promote biofilm growth at 37°C during 48 h. After this period, irradiation of the experimental groups was done. The irradiated and non-irradiated biofilms were analyzed 15 h after incubation. The experiment was performed in triplicate at different moments.

Irradiation

The LLL source used in this study was a near infrared indium gallium arsenide phosphide (InGaAsP) diode laser prototype (LASERTable; 780 ± 3 nm wavelength, 0.4 W power output). This device has been specifically designed to provide a uniform irradiation of the wells of culture plates in which cells or microorganisms are seeded, according to the parameters established for each study. The radiation originated from the LASERTable was delivered on the base of each 24-well plate at doses of 5, 10 and 20 J/cm² with irradiation times of 250, 500 and 1000 s, respectively. The laser doses were established according to Nussbaum et al. (4), who evaluated the bactericidal capacity of LLLT with different wavelengths.

After irradiation, the plates with the biofilms formed in each well were incubated at 37°C for 15 h, and then the microorganisms were subjected to the MTT assay for analysis of succinic dehydrogenase (SDH) enzyme production, quantification of biofilm growth

(cfu/mL) and analysis cell morphology under scanning electron microscopy (SEM). The microorganisms assigned to the control groups received the same treatment as that of the experimental groups. The 24-well plates containing the control microorganisms were maintained in the LASERTable for the same irradiation times used in the respective experimental groups, though without activating the laser source (sham irradiation).

Analysis Biofilm Viability (MTT Assay)

Previous studies have already determined microorganisms viability by MTT assay (16,17). As biofilms are capable of aerobic respiration which is held by cytosolic enzymes (18) the MTT assay was carried out to evaluate the viability of the biofilms after irradiation as described by Kwieciński et al. (16).

For this method, twelve specimens of each experimental and control group were used for analysis of cell metabolism 15 h after the active or sham irradiation. Cell metabolic activity was evaluated by SDH production, which is a measure of the respiratory metabolism of the cells. Each well containing the biofilms received 300 μ L of MTT solution (5 mg MTT salt in 1 mL PBS). The plates were incubated at 37°C for 2 h. Thereafter, the MTT solution was carefully aspirated from each well and replaced by 300 μ L of acidified isopropanol solution (0.04 N HCl) to dissolve the violet formazan crystals resulting from the cleavage of the MTT salt ring by the SDH enzyme produced by cellular aerobic respiration of viable microorganisms. After agitation and confirmation of the homogeneity of the solutions, 3 aliquots de 100 μ L of each well were transferred to a 96-well plate (Costar Corp., Cambridge, MA, USA). The respiratory metabolism was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Multiskan Ascent 354; Labsystems CE, Lês Ulis, France). The values obtained from the 3 aliquots were averaged to provide a single value. The absorbance was expressed in numeric values, which were subjected to statistical analysis to determine the respiratory activity on biofilms.

Biofilm Quantification

Quantification of biofilm was done to evaluate the effect of LLLT on the growth of the microorganisms. Fifteen hours after irradiation, the biofilms were

washed twice with sterile PBS to remove non-adhered microorganisms. Then, 1 mL of PBS was added to each well and the adhered biofilm was carefully scraped (15). The whole procedure was performed by a single operator following a standardized protocol. The suspension containing the microorganisms was aspirated and transferred to a tube, which was vortexed during 1 min. Serial dilutions of the final suspension (10^{-1} to 10^{-4}) were prepared and inoculated in sucrose bacitracin agar (SB₂₀) and Sabouraud dextrose agar (SDA) for growth of *S. mutans* and *C. albicans*, respectively. The plates were incubated at 37°C during 48 h, and the biofilm counts were expressed as cfu/mL.

Analysis of Cell Morphology by SEM

Two specimens of each experimental and control group were used for analysis of the morphology and number of microorganisms in the biofilms 15 h after the active or sham irradiation. Sterile 13-mm-diameter cover glasses (Fisher Scientific) were placed on the bottom of the wells of all groups immediately before seeding of the microorganisms. After exposure to the experimental conditions, the culture medium was removed and the viable cells that remained adhered to the glass substrate were fixed in 1 mL of buffered 2.5% glutaraldehyde solution during 2 h. Next, the cells were submitted to three 5-min rinses with 1 mL PBS, post-fixed in 200 μ L of 1% osmium tetroxide during 60 min. The microorganisms adhered to the glass substrate were then dehydrated in a series of increasing ethanol concentrations [30%, 50%, 70%, 95% (twice) and 100% (twice) - 30 min in each solution], immersed in 1,1,1,3,3,3-hexamethyldisilazane (HMDS; Acros Organics, Springfield, NJ, USA) for 90 min, and stored in a desiccator during 12 h. The cover glasses were then mounted on metallic stubs, sputter-coated with gold and the morphology of the surface-adhered microbial cells was examined with a scanning electron microscope (JEOL-JMS-T33A Scanning Microscope; USA Inc., Peabody, MA, USA).

Statistical Analysis

Data from SDH production and biofilm quantification (cfu/mL) had a non-normal distribution (Kolmogorov-Smirnov, $p < 0.05$) and were analyzed by Kruskal-Wallis and Mann-Whitney non-parametric tests. A significance level of 5% was set for all analyses.

RESULTS

Analysis of Biofilm Respiratory Activity (MTT Assay)

SDH production data (MTT assay) according to type of biofilm and laser dose are presented in Table 1. For all laser doses, the lowest SDH production was observed for *S. mutans* (SSB) ($p < 0.05$) followed by *C. albicans* (SSB) and DSB. No significant difference ($p > 0.05$) was found between *C. albicans* (SSB) and DSB.

LLLT did not have significant influence on SDH production by *C. albicans* (SSB) and DSB, regardless of

the laser dose ($p > 0.05$). However, irradiation of *S. mutans* (SSB) caused significant reduction of SDH production ($p < 0.05$) at all laser doses, without significant difference among them ($p > 0.05$). In other words, regardless of the dose, LLLT influenced the respiratory activity of *S. mutans* (SSB).

Biofilm Quantification

Quantification of biofilm (cfu/mL), according to the type of biofilm and laser dose (J/cm^2) is presented in Table 2. There was a significant difference ($p < 0.05$) in the reduction in the growth of *S. mutans* (SSB) between the control and irradiated groups; in the groups subjected to LLLT, the reduction was more accentuated and occurred in a dose-dependent fashion.

Regarding DSB, the 5 J/cm^2 laser dose promoted a significant reduction in the growth of *S. mutans*, and this reduction was only slightly intensified when higher laser doses (10 and 20 J/cm^2) were used. In association with *C. albicans* in a DSB configuration, *S. mutans* exhibited a more intense growth and greater resistance to LLLT compared to the yeast.

Table 1. SDH enzyme production (MTT assay) according to the type of biofilm and laser dose (J/cm^2).

Irradiation dose (J/cm^2)	Microorganism		
	<i>S. mutans</i>	<i>C. albicans</i>	<i>S. mutans</i> + <i>C. albicans</i>
0 (control)	1.31 (1.27-1.32) ^{A b}	2.44 (2.37-2.59) ^{A a}	2.47 (2.29-2.55) ^{A a}
5	1.01 (0.93-1.08) ^{B b}	2.50 (2.20-2.68) ^{A a}	2.38 (2.02-2.69) ^{A a}
10	1.02 (0.75-1.27) ^{B b}	2.47 (2.07-2.65) ^{A a}	2.65 (1.91-2.69) ^{A a}
20	0.96 (0.81-1.12) ^{B b}	2.43 (2.17-2.73) ^{A a}	2.49 (1.81-2.66) ^{A a}

Values expressed as medians of SDH production (P25-P75) (n=12) (optical density). Same uppercase letters in columns and lowercase letters in rows indicate no statistically significant difference (Mann-Whitney, $p > 0.05$).

Table 2. Quantification of biofilm (cfu/mL) according to the type of biofilm and laser dose (J/cm^2).

Irradiation dose (J/cm^2)	Biofilm (cfu x 10 ⁷)			
	<i>S. mutans</i> SSB	<i>C. albicans</i> SSB	DSB (<i>S. mutans</i>)	DSB (<i>C. albicans</i>)
0 (control)	0.20(0.19-0.20) ^{A c}	0.58 (0.47-0.65) ^{A a}	0.41 (0.39-0.44) ^{A b}	0.40 (0.34-0.44) ^{A b}
5	0.09 (0.08-0.09) ^{B c}	0.28 (0.28-0.29) ^{C b}	0.35 (0.32-0.38) ^{B a}	0.34 (0.20-0.38) ^{B ab}
10	0.07 (0.05-0.07) ^{C c}	0.23 (0.20-0.31) ^{C b}	0.40 (0.36-0.43) ^{AB a}	0.21 (0.15-0.27) ^{C b}
20	0.02 (0-0.03) ^{D c}	0.39 (0.31-0.48) ^{B a}	0.39 (0.30-0.46) ^{AB a}	0.15 (0.14-0.15) ^{D b}

Values expressed as medians (P25-P75) (n=9) of the number of cfu x 10⁷. Same uppercase letters in columns and lowercase letters in rows indicate no statistically significant difference (Mann-Whitney, $p > 0.05$). SSB = single-species biofilm. DSB = dual-species biofilm.

Irradiation of *C. albicans* (SSB) with doses of 5 and 10 J/cm² resulted in a statistically significant reduction of growth compared to the non-irradiated control. Increase of laser dose to 20 J/cm² produced only a slight intensification in biofilm growth inhibition compared to the lower doses, though significant when compared to the control group.

In association with *S. mutans* in a DSB configuration, *C. albicans* presented a statistically significant growth inhibition in the irradiated groups compared to control group, and this reduction was dose-dependent.

Comparing the behavior of the 3 types of biofilms,

it can be observed that when the microorganisms were associated in a DSB configuration, *C. albicans* was more susceptible to LLLT at the highest laser dose (20 J/cm²).

Biofilm Morphology

Figure 1 illustrates the effects of LLLT on *S. mutans* (SSB). The irradiation promoted a decrease in the number of microorganisms, though without significant difference among the laser doses or the types of biofilm. Regarding the morphological alterations, there were no difference between the control group (Fig. 1A) and irradiated groups (Fig. 1 B-D).

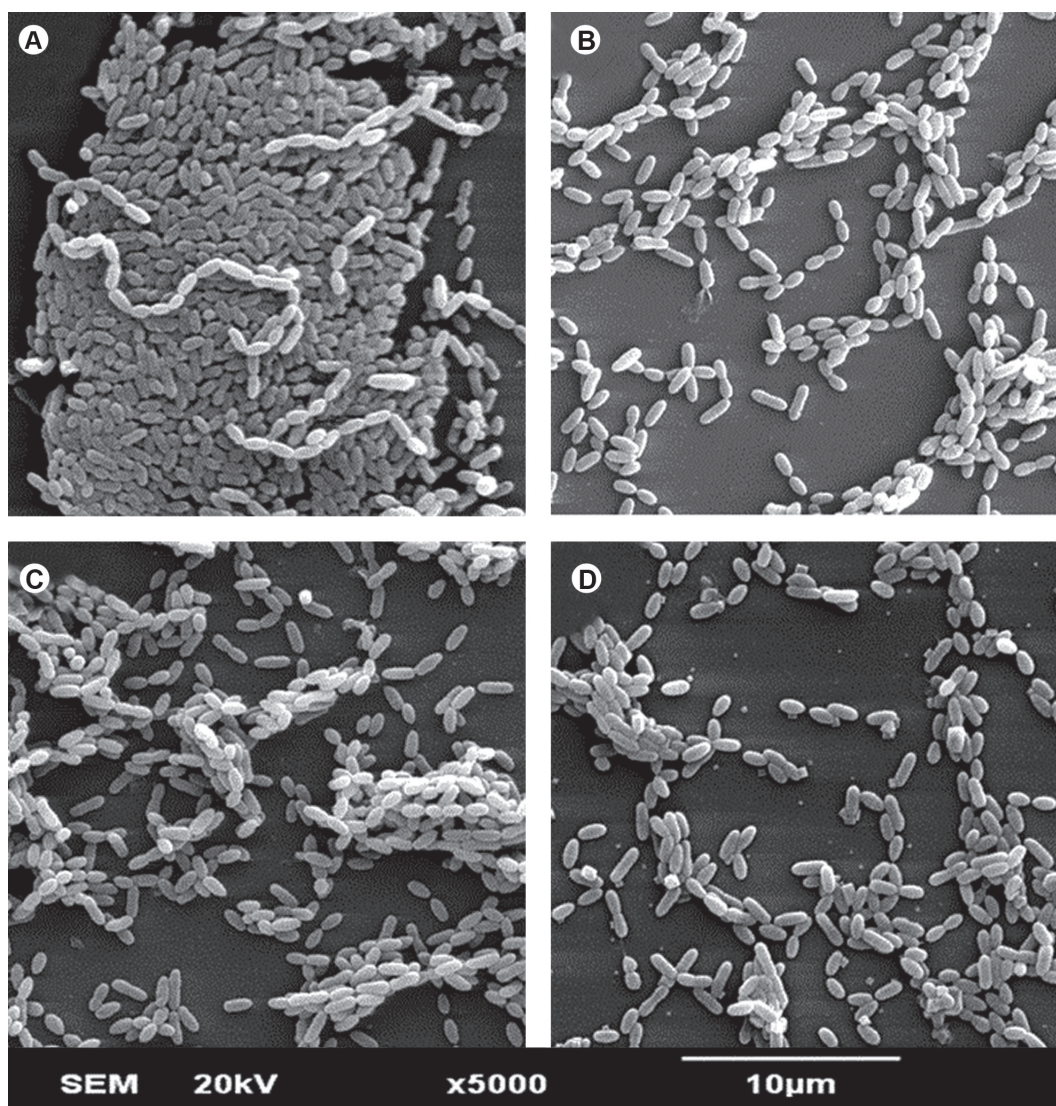


Figure 1. SEM micrograph showing the morphology and structure of SSB of *S. mutans* (Original magnification $\times 5000$). A= Control, B-D= Biofilm after irradiation with laser dose of 5, 10 and 20 J/cm².

Figures 2 and 3 illustrate the effects of LLLT on the morphology and structure of single-species biofilms of *C. albicans* and DSB, respectively. Regardless of laser dose, irradiation of *C. albicans* (SSB) did not cause morphological alterations in the yeasts nor affected the number of microorganisms that remained adhered to glass substrate (Fig. 2). On the other hand, Figure 3 clearly shows that the association of microbial species caused a reduction in the formation of *C. albicans* hyphae.

DISCUSSION

Little research has been done on the formation

and behavior of multi-species biofilms subjected to different treatment protocols. Understating inter-species interaction is necessary because this relationship seems to exert a significant influence on the behavior of the species (19). The present study evaluated the effect of LLLT (780 nm) on SSB and DSB of *S. mutans* and *C. albicans*, which are typical oral microorganisms (20) and are involved in the pathogenesis of diseases of high incidence, such as caries and candidiasis (14). The protocols adopted in the present study showed a significant reduction in SDH production when *S. mutans* (SSB) was irradiated with a LLLL emitting at 780 nm. This effect is probably related to a decrease in the number

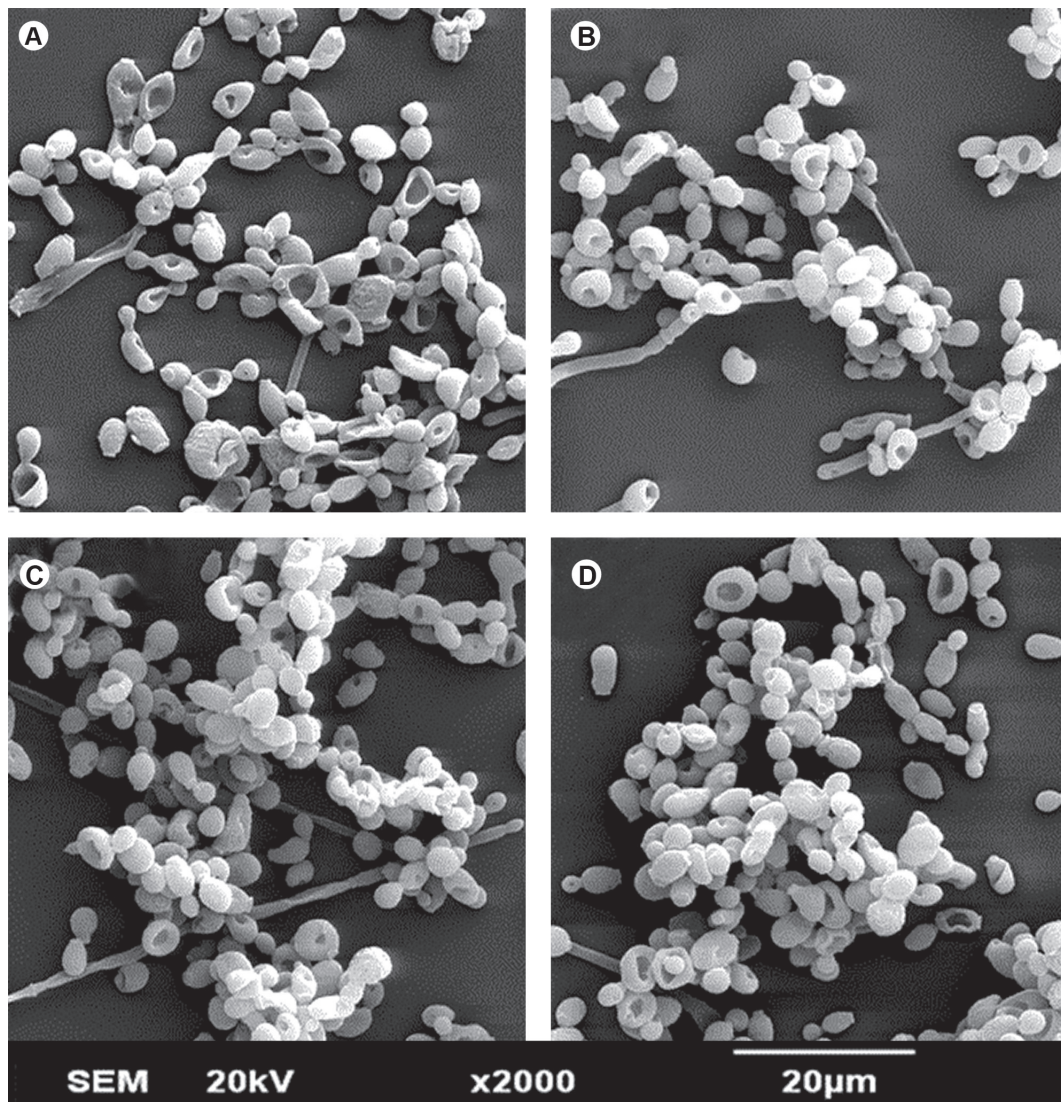


Figure 2. SEM micrograph showing the morphology and structure of SSB of *C. albicans* (Original magnification $\times 2000$). A= Control, B-D= Biofilm after irradiation with laser dose of 5, 10 and 20 J/cm².

of microorganisms in the biofilms, as demonstrated by biofilm growth (cfu/mL) and SEM analysis. The decrease in the number of biofilm microorganisms was similar for all laser doses (5, 10 and 20 J/cm²), indicating that the effect of laser on *S. mutans* was not dose-dependent. Nussbaum et al. (4) also demonstrated that LLLT ($\lambda=810$ nm) at 5, 10, 20 and 50 J/cm² doses was effective in inhibiting growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, differently from our findings for *S. mutans*, those authors found dose- and species-dependent results (4,5). This variation in the responses to the treatment could be attributed to the fact that the different microbial species used in the studies

might have different susceptibility to LLLT (5). In the present study, counting of cfu/mL of *S. mutans* biofilms showed a significant inhibition in the growth of this microorganism after irradiation, in a dose-dependent manner. This relationship can be derived from an early action of LLLT by inhibiting biofilm growth, possibly due to a disaggregation of microorganisms caused by the exposure of these microorganisms to laser (8). Data referring to *S. mutans* (SSB) quantification differ from those of cell respiration, which can be affected in a later moment due to the intracellular stress promoted by LLLT (8). These different effects of LLLT on *S. mutans* may justify the fact that the results of SDH production and

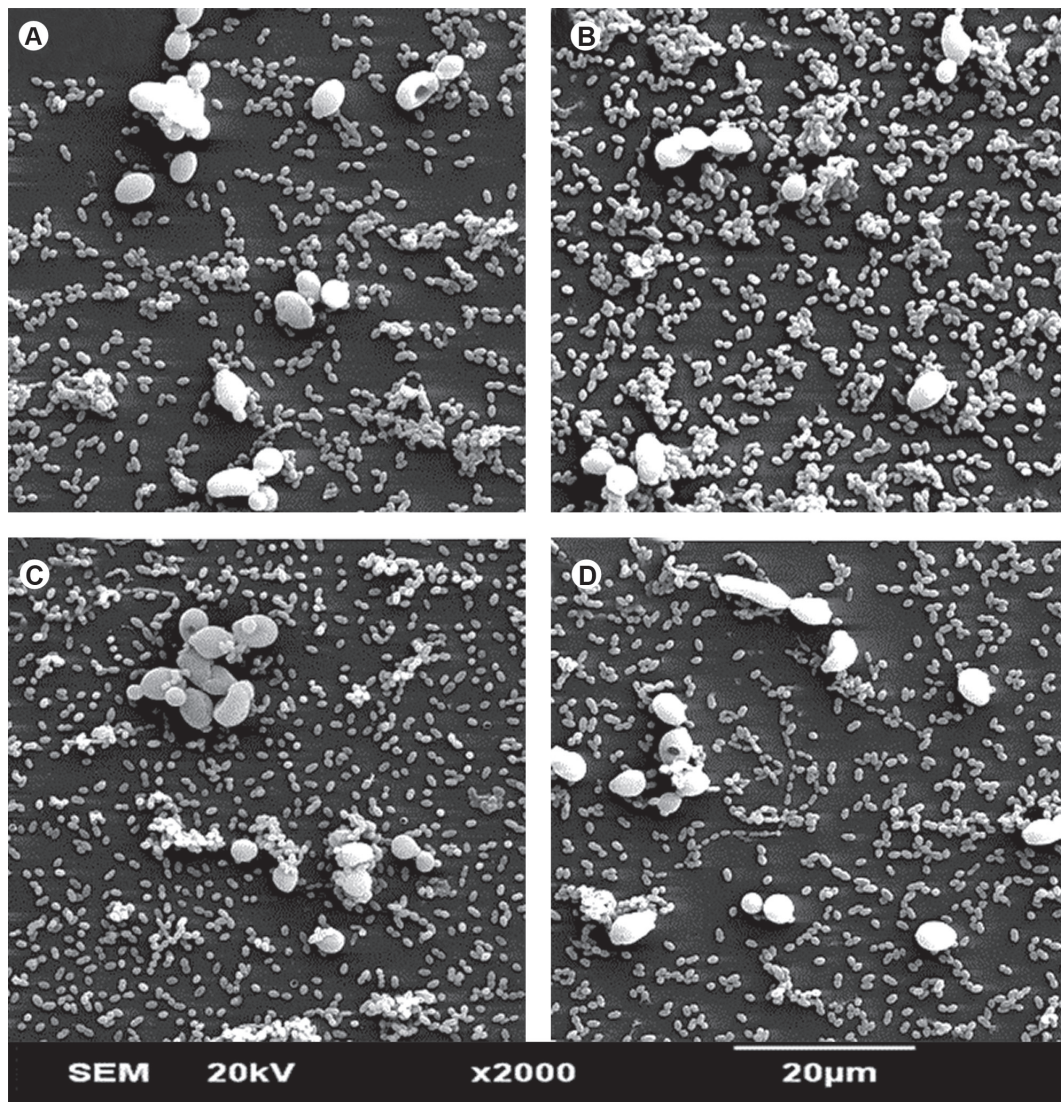


Figure 3. SEM micrograph showing the morphology and structure of DSB (*S. mutans* plus and *C. albicans*) (Original magnification $\times 2000$). A= Control, B-D= Biofilm after irradiation with laser dose of 5, 10 and 20 J/cm².

cfu counts were not observed in the same intensity for this microorganism.

Regarding the morphology of irradiated microorganisms, there were no morphological alterations in the microorganisms after LLLT. However, the analysis of SEM micrographs showed a decrease in the number of *S. mutans* adhered to the glass substrate in the irradiated groups compared to the group control. This result suggests that LLLT might have altered biofilm organization, leading to the loss of microbial adherence to the substrate, as demonstrated by Krespi et al. (10). Those authors observed a disaggregation of microorganisms after irradiation of *S. aureus* biofilms with a LLL emitting at 810 nm. Although there is no evidence of induction of cell death, irradiation in the parameters used in the present study seems to have promoted disorganization and disaggregation of the microorganisms in the biofilm, inhibiting their growth and metabolism.

The association of the microbial species in a DSB configuration seemed to have had a protective effect for *S. mutans*, since no reduction in SDH production or in the number and growth of this microorganism was observed. The interaction between *C. albicans* and *S. mutans* biofilms has been characterized as a mutualistic relationship, which is beneficial for both species (21). The yeast may offer a physical scaffold for the proliferation of *S. mutans*, thus increasing numbers of this bacterium in the biofilm (21). It has also been demonstrated an increase in the growth of *C. albicans* when associated with *S. mutans*. This finding confirms not only mutualism, but also coaggregation, which increases the adhesion of both microbial species to the biofilm (12).

In the present study, LLLT was not effective in reducing SDH production in *C. albicans* (SSB). However, irradiation with a dose of 5 J/cm² inhibited the growth of this microorganism. In the same way as for the *S. mutans* (SSB), this result indicates the laser may cause early or later effects on this yeast. The reduction of cell respiration may be one of the later effects caused by LLLT (8).

The irradiation of DSB of *C. albicans* and *S. mutans* inhibited the growth (cfu/mL) of the yeast without reducing its SDH production. Regarding the morphology, the interaction of the microbial species promoted a reduction in the formation of *C. albicans* hyphae. This effect has also been recently described by Jarosz et al. (20), who suggested an inhibitory effect of *S.*

mutans over *C. albicans* by the release of acid molecules in the environment. The presence of these molecules in the environment seems to inhibit the formation and maturation of *C. albicans*. Some authors investigating the interaction between these microorganisms in biofilm formation have suggested that *C. albicans* inhibition in the presence of *S. mutans* in multi-species biofilms is due to competition for adhesion niches and nutrition, thus decreasing the adhesion of these microorganisms when associated (11).

Under the conditions tested in the present study, LLLT had an inhibitory effect on typical oral microbial biofilms, and this capacity can be altered according to the interactions between different species. Further research is needed to evaluate the behavior of different microorganisms typically found in the oral cavity, and their interaction in biofilms when subjected or not to LLLT.

RESUMO

O objetivo deste estudo foi avaliar o efeito de parâmetros específicos de irradiação com laser de baixa intensidade sobre biofilmes formados por *Streptococcus mutans* (*S. mutans*), *Candida albicans* (*C. albicans*) ou associação de ambas as espécies. Biofilmes isolados ou associados destes microrganismos foram irradiados com um dispositivo laser infra-vermelho próximo de diodos InGaAsP (LaserTABLE 780 ± 3 nm, 0,04W), utilizando-se para isto o dispositivo LASERTable. Quinze horas após a irradiação, foi demonstrado, por meio da avaliação da viabilidade celular (Teste de MTT), da morfologia das células (MEV) e do crescimento do biofilme (UFC/mL), que esta terapia foi capaz de reduzir o metabolismo celular, número de microrganismos presentes no biofilme, bem como seu crescimento no local. Quanto à viabilidade celular, a resposta à irradiação do biofilme de *S. mutans* (SSB) foi semelhante para todas as doses de energia, sendo que o crescimento do biofilme foi dose dependente. Porém, quando associado à *C. albicans*, este microrganismo apresentou resistência à fototerapia. Já a *C. albicans* associada ao *S. mutans* apresentou redução de crescimento significativa, sendo este resultado também foi dose dependente. A morfologia dos microrganismos não foi alterada pelas irradiações realizadas quando em biofilmes isolados. A associação entre os microrganismos promoveu redução na formação de hifas pela *C. albicans*. A laserterapia de baixa intensidade apresentou efeito inibitório sobre microrganismos, sendo que esta capacidade pode ser alterada de acordo com a interação entre diferentes microrganismos.

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