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Light-induced effects against *Candida albicans* and *Staphylococcus aureus*

Luz produz efeito sobre *Candida albicans* e *Staphylococcus aureus*

Efectos inducidos por la luz contra *Candida albicans* y *Staphylococcus aureus*

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Abstract
Objectives: The present study evaluated the action of LED light sources at different wavelengths on cell viability, lipid peroxidation, and Top I and II gene expression of *Candida albicans* and *Staphylococcus aureus*. Methods: Planktonic cultures were subjected of illumination, and the post- irradiation cell proliferation was assessed by quantification of metabolic mitochondrial activity. Next, the response of the microorganisms to the treatments was assessed. Results: Cell viability (CFU/mL) reduction occurred only for the fungus by 0.5 and 0.6 $\log_{10}$ using yellow LED 0.1 and 10 J/cm^2, respectively. For *S. aureus*, none of the evaluated wavelengths reduced cell viability versus the control. Production of intracellular ROS occurred in all tested light doses and wavelengths, except for 0.1 J/cm^2 of yellow LED for both microorganisms. Lipid peroxidation occurred only for *C. albicans* after exposure to 10 J/cm^2 of yellow LED, 15 and 50 J/cm^2 of blue LED, and 300 and 500 lux of white LED. The two doses of blue light and red light decreased the expression of TOP II of *C. albicans* and TOP I of *S. aureus*. The two doses of yellow and white lights promoted an increase in the expression of the genes that encode TOP II and TOP I for both species. Conclusion: The results demonstrated that the mechanisms of action of white LEDs and at blue (455 nm), red (660 nm) and yellow (590 nm) wavelengths seem to be related to the production of ROS, lipid peroxidation, and DNA damage.
Keywords: Phototherapy; *Candida albicans*; *Staphylococcus aureus*; Oxidative Stress; Lipid peroxidation.

Resumo
Objetivos: O presente estudo avaliou a ação de fontes de luz LED em diferentes comprimentos de onda na viabilidade celular, peroxidação lipídica e expressão gênica de Top I e II de *Candida albicans* e *Staphylococcus aureus*. Métodos: Culturas planctônicas foram submetidas à iluminação e a proliferação celular pós-irradiação foi avaliada pela quantificação da atividade metabólica mitocondrial. Em seguida, avaliou-se a resposta dos microrganismos aos tratamentos. Resultados: A redução da viabilidade celular (UFC/mL) ocorreu apenas para o fungo em 0,5 e 0,6 $\log_{10}$ utilizando LED amarelo 0,1 e 10 J/cm^2, respectivamente. Para *S. aureus*, nenhum dos comprimentos de onda avaliados reduziu a viabilidade celular em relação ao controle. A produção de ROS intracelular ocorreu em todas as doses de luz e comprimentos de onda testados, exceto 0,1 J/cm^2 de LED amarelo para ambos os microrganismos. A peroxidação lipídica ocorreu apenas para *C. albicans* após exposição a 10 J/cm^2 de LED amarelo, 15 e 50 J/cm^2 de LED azul e 300 e 500 lux de LED branco. As duas doses de luz azul e luz vermelha diminuíram a expressão de TOP II de *C. albicans* e TOP I de *S. aureus*. As duas doses de luzes amarela e branca promoveram aumento na expressão dos genes que codificam TOP II e TOP I para ambas as espécies. Conclusão: Os resultados demonstraram que os

mecanismos de ação dos LEDs brancos e nos comprimentos de onda azul (455 nm), vermelho (660 nm) e amarelo (590 nm) parecem estar relacionados à produção de EROs, peroxidação lipídica e danos ao DNA.
Palavras-chave: Fototerapia; *Candida albicans*; *Staphylococcus aureus*; Estresse Oxidativo; Peroxidação lipídica.

Resumen

Objetivos: El presente estudio evaluó la acción de fuentes de luz LED a diferentes longitudes de onda sobre la viabilidad celular, la peroxidación lipídica y la expresión génica Top I y II de *Candida albicans* y *Staphylococcus aureus*. Métodos: Los cultivos planctónicos se sometieron a iluminación y la proliferación celular posterior a la irradiación se evaluó mediante la cuantificación de la actividad mitocondrial metabólica. Se evaluó la respuesta de los microorganismos a los tratamientos. Resultados: La reducción de la viabilidad celular (UFC/mL) ocurrió solo para el hongo en 0,5 y 0,6 $\log_{10}$ usando LED amarillo 0,1 y 10 J/cm^2, respectivamente. Para *S. aureus*, ninguna de las longitudes de onda evaluadas redujo la viabilidad celular frente al control. La producción de ROS intracelular se produjo en todas las dosis de luz y longitudes de onda probadas, a excepción de 0,1 J/cm^2 de LED amarillo para ambos microorganismos. La peroxidación de lípidos ocurrió solo para *C. albicans* después de la exposición a 10 J/cm^2 de LED amarillo, 15 y 50 J/cm^2 de LED azul y 300 y 500 lux de LED blanco. Las dos dosis de luz azul y luz roja disminuyeron la expresión de TOP II de *C. albicans* y TOP I de *S. aureus*. Las dos dosis de luz amarilla y blanca promovieron un aumento en la expresión de los genes que codifican TOP II y TOP I para ambas especies. Conclusión: Los resultados demostraron que los mecanismos de acción de los LED blancos y en las longitudes de onda azul (455 nm), rojo (660 nm) y amarillo (590 nm) parecen estar relacionados con la producción de ROS, la peroxidación lipídica y el daño del DNA.
Palabras clave: Fototerapia; *Candida albicans*; *Staphylococcus aureus*; Estrés oxidativo; Peroxidación lipídica.

1. Introduction

Nosocomial infections are secondary infections acquired during the treatment of other systemic conditions, especially in hospitalized patients (Destaedt et al.,2018). These infections have high mortality and morbidity rates, generating high costs for the public health sector and are associated with the development of multidrug-resistant pathogenic microorganisms to conventional therapies [Magill et al.,2014). *Candida albicans* and *Staphylococcus aureus* are the most prevalent microorganisms in this type of infection (Destaedt et al.,2018). These microorganisms live commensally in the human mucosa and skin, and, in situations of imbalance, they can act as opportunistic pathogens, infecting, invading, and replicating in the human body (Zeina et al., 2001). Hence, the search for alternative and effective modalities to combat microorganisms that do not promote resistance is necessary. Among these new approaches are the technologies based on the application of light (Silbert & Milburn,2000; Costa et al., 2010; Rosario et al., 2012).

Phototherapy employing light-emitting diodes (LED) has the potential for many applications in the medical-dental area. They are economical lamps, with less energy consumption and lower thermal productivity that minimizes damage to tissues. Additionally, compared to the laser, LEDs are low-cost, portable, and easy to handle systems (Silbert & Milburn,2000; Costa et al., 2010; Rosario et al., 2012). The mechanism of action that promotes the inactivation of microorganisms after phototherapy is not yet established. However, it generally accepted hypothesis is that light acts by exciting endogenous chromophores present in microbial cells, such as porphyrins and iron-free flavins, resulting in reactive oxygen species (ROS) [Dai et al., 2012; Wang et al., 2017). The process of cellular inactivation is similar to what occurs in photodynamic therapy. On the other hand, phototherapy is attractive because it does not require any added photosensitizers, does not involve possibly harmful ultraviolet radiation, and inactivates microbial cells, regardless of their antibiotic resistance status.

The antimicrobial activity of blue light has been evaluated at different wavelengths [Ganz et al., 2005; Guffey & Wilborn, 2006; Enwemeka et al., 2008; Masson-Meyers et al.,2015; Feuerstein et al., 2004; Soukos et al., 2005; Fontana et al., 2015). Blue light has a bactericidal effect reducing the cell viability of *Helicobacter pylori* (Ganz et al., 2005); *S. aureus*, susceptible and resistant to methicillin (Guffey & Wilborn, 2006; Enwemeka et al., 2008; Masson-Meyers et al.,2015); *Pseudomonas aeruginosa* (Guffey & Wilborn, 2006); and in bacteria associated with periodontal disease (Feuerstein et al., 2004; Soukos et al., 2005; Fontana et al., 2015). Also, the blue LED (455 nm) caused a genotoxic effect on *C. albicans*

(Carmello et al.,2015). In contrast, the red LED did not show antimicrobial activity on the microorganisms *Porphyromonas gingivalis*, *S. aureus*, and *Escherichia coli* (Feuerstein et al., 2004; Nussbaum et al.,2002; Kim et al., 2013). In addition, there are no reports in the literature on the action of white and yellow LEDs on microbial inactivation. Thus, evaluating the antimicrobial activity of different wavelengths of LED light and its mechanism of action is of great relevance. Therefore, the present study aimed to evaluate the action of LED light sources at different wavelengths (blue, red, white, and yellow) on cell viability, lipid peroxidation, and Top I and II gene expression of *S. aureus* and of *C. albicans*, respectively.

2. Methodology

Cultivation of microorganisms

The strains of *C. albicans* (ATCC 90028) and *S. aureus* (MSSA ATCC-25923) were evaluated. *C. albicans* was reactivated on YEPD (Sigma-Aldrich) agar and maintained at 37 ºC for 48 h. It was then inoculated in YNB (Yeast Nitrogen Base; Difco) and incubated at 37 ºC during the overnight period (16 h). Next, the volume of 500 uL was resuspended in the same culture medium at 1:20 dilution, followed by incubation at 37 ºC, until the *C. albicans* culture reached half of the exponential growth phase (mid-log ~ 10^6 CFU/mL - DO_{540nm}). The standardized suspensions were centrifuged and washed 3X with phosphate-buffered saline solution (PBS, pH 7.2) at 4000 x*g* for 5 min, to be used in the experiments.

S. aureus was reactivated on BHI agar plates (Brain Heart Infusion Agar) supplemented with amphotericin B (Altieri et al., 2012) (Acumedia Fabrica Inc., Baltimore, MD, USA) during 48 h. Next, the microorganism was inoculated in TSB medium (Tryptic Soy Broth-Acumedia Fabrica Inc., Baltimore, MD, USA) and incubated at 37 °C overnight (18 h). Next, an aliquot of the suspension was adjusted to a 1:20 concentration using the same culture medium. It was again incubated at 37 °C until the culture reached half of the exponential growth phase (mid-log ~ 10^7 CFU/mL - DO_{600nm}). Next, the standardized suspensions were centrifuged and washed 3X with PBS at 4000 x *g* for 5 min, to be used in each experiment.

The standard planktonic cultures of *C. albicans* and *S. aureus* were aliquoted in 96-well plates and subjected to the proposed treatments.

Screening and evaluation by quantification of metabolic mitochondrial activity (XTT)

The use of LEDs in domestic and public via have increased in the last 20 years. In addition, the LED light has been used as a light source for medical applications. Therefore, a screening composed of different doses of energy of each LED light (Table 1) was carried out in order to investigate which dose would be able to alter the mitochondrial activity of microorganisms (*C. albicans* and *S. aureus*). For this, standardized suspensions of microorganisms were exposed to LED light using a Biotable (Biotable / MMOptics COD / 10.02546 - MMO LTDA - São Carlos, Brazil) consisting of 24 LEDs positioned to radiate the base of the plates where the cultures were grown homogeneously and individually. Each irradiation was performed at a standardized distance of 2.5 cm. After treatments, the quantification of metabolic mitochondrial activity of the microorganisms was assessed by performing the XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium5-carboxanilide] assay. XTT is a yellow tetrazolium salt and its colorimetric reaction is interpreted as the cellular metabolism of the microorganism (Gabrielson et al., 2002; Khot et al.,2008). The result of this chemical reaction was obtained using the spectrophotometer with a 492 nm filter (Silva et al., 2010). The experiment was performed on three separate occasions in quadruplicate (n = 12).

Table 1. Description of the parameters used in the screening of LED light on suspensions of *C. albicans* and *S. aureus*.

LED	Intensity (mW/cm^2)	Energy dosage
Blue (455 nm)	30	8; 15; 37.5; 50; 60; 100 J/cm^2
Red (660nm)	71	1; 5; 25; 37.5; 50; 100 J/cm^2
Yellow (590)	12	0.1; 0.5; 1; 2.5; 5; 10 J/cm^2
White (multicromatic)	21	300; 400; 500 lux

Source: Authors.

Based on the metabolic mitochondrial activity results, the light parameters were selected to be evaluated in the next stages of the study. For the parameters that resulted in a significant change in mitochondrial activity of the microorganisms, the response of then to the lights doses was assessed using the following tests: cell viability via CFU/mL; analysis of oxidative stress (detection of increased production of ROS with the redox-sensitive fluorochrome dichlorofluorescein diacetate), analysis of lipid peroxidation (using the Fox method) (Devasagayam et al., 2003) and analysis of gene expression of *C. albicans* Top II and *S. aureus* Top I. In addition, the lower dose that do not resulted in change in the metabolism were evaluated as comparison parameters.

Serial dilution and plating of planktonic cultures

The number of adhered cells was determined by counting colony-forming units. Immediately after the treatments, 100 µL aliquots of microorganisms from each well were subjected to serial dilutions (10^{-4} to 10^{-7}) in PBS (pH 7.2). The number of *C. albicans* was determined by plating in duplicate in Petri dishes containing Sabouraud Dextrose Agar plates (SDA—Acumedia Manufactures Inc., Baltimore, MD, USA) with chloramphenicol. The same procedures were performed for the *S. aureus*, which were plated on Mannitol Salt Agar (Acumedia Manufactures Inc., Baltimore, MD, USA) . All plates were incubated aerobically at 37°C for 48 h and the values of CFU mL^{-1} were calculated.

Analysis of oxidative stress

This test assessed the oxidative stress undergone by microbial cells and measure cellular ROS produced after treatments. The cells were cultured, adjusted to a concentration of $1.0x10^6$ cells/mL, and suspended in PBS. Next, 50 µl of untreated cells were aliquoted into microtubes to assess ROS production. The 2',7'-dichlorofluorescein diacetate probe (H_2DCFDA; Sigma-Aldrich) was added to a final concentration of 50 mM and the microtubes were incubated for 1 h in an orbital shaking (30 ºC -120 rpm) in the dark. After this period, the cells were centrifuged at 4000 x*g* for 5 min and washed 2X with PBS, transferred to a 96-well plate, and then subjected to the proposed treatments. The group containing hydrogen peroxide (H_2O_2) was added to the analysis as a control group to compare the ROS production. For that, the cells were incubated at 30 °C at 120 rpm with 10 mM H_2O_2 for 20 min (Carmello et al., 2015). The fluorescence of the cells was measured in the Fluoroskan (Thermo Fisher Scientific, Inc.) using the recommended spectral range of excitation (485 nm) and emission (538 nm).

Analysis of lipid peroxidation (FOX method)

The products of the damage produced by oxidative stress was measured by TBARS (Thiobarbituric acid reactive substances). The cells were centrifuged and washed twice with 20 mM Tris/HCl buffer, pH 7.4, to assess lipid peroxidation. The pellet was suspended in 500 ml of the same buffer, containing 10% trichloroacetic acid, and 1.5 g of glass beads were added. Cells were broken by performing 3 cycles of 1 min vortexing, followed by 1 min on ice. The supernatants obtained after centrifugation at 4000 x *g* for 5 min were mixed with 0.1 ml of 0.1 M EDTA and 0.6 ml of thiobarbituric acid (TBA; 1% w/v) in 0.05 M NaOH. The mixture was incubated in a boiling water bath for 20 min and, after cooling down, the absorbance was measured at 532 nm.

Analysis of expression of the genes that encode enzymes related to DNA repair

Top 1somerase II of *C. albicans* and Top 1somerase I of *S. aureus*

After the treatments, the colonies were recovered and stored in 300 µl glycerol (50%; Labsynth, Diadema, São Paulo, Brazil) at -80 ºC until RNA extraction, synthesis of cDNA and qPCR.

RNA extraction and purification

The RNA of the samples was extracted using the phenol-chloroform separation method and purified via treatment with column DNA (Qiagen) and in solution (TURBO DNase; Ambion) (Cury & Koo,2007). DNAse was removed using the RNeasy MinElute clean-up kit (Qiagen), following the manufacturer's recommendations. The purified RNA's integrity was determined by 1% agarose gel electrophoresis (Life Technologies, Carlsbad, CA, United States) containing 0.3 µg/mL ethidium bromide. Spectrophotometric reading was performed to assess the amount (OD_{260nm}) and purity ($OD_{260/280}$ ratio) of the total RNA (DS-11 + Nano-spectrophotometer, Denovix, Wilmington, DE).

Synthesis of cDNA

The cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, CA, United States). For +RT cDNA synthesis, 1 µg of total RNA was mixed with 4 µL of the 5X iScript buffer, 1 µL of the reverse transcriptase enzyme, and q.s.p. complete 20 µL. The reactions were incubated on the CFX96 Touch ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States) with the cycle recommended by the manufacturer (25 ºC/5 min, 42 ºC/30 min, 85 ºC/5 min, finalized with 4 ºC/∞). Negative control (-RT) was synthesized using molecular water instead of reverse transcriptase to check for DNA contamination. The cDNA samples were stored at -20 ºC until the moment of gene expression quantification.

Quantification of gene expression using RT-qPCR

For the RT-qPCR reaction, primers for the *C. albicans* TOP II and *S. aureus* TOP I genes (Table 2) were designed and standardized in the present study.

Table 2. Primers used in the present study.

Gene	Primer sequence (F: forward; R: reverse)	Tm (°C)	Product length (bp)	Optimal concentration (nM)
TOP II- *C. albicans*	F – TCAGAGATCCCAGTATGAAG	58	249	350
	R – CCACTTCAAATTGGGTAGAG			
TOP I- *S. aureus*	F – TTACAACAGAAGGGCGTACA	57	171	300
	R – CGTGCATTTTCGTATGAATG			

Source: Authors.

Primers were purchased from ThermoFisher (Life Technologies Brasil Com Ind Prod Bio LTDA, São Paulo, SP, Brazil). The amplification of the target sequences was performed by conventional PCR methods using the *Taq* DNA Polymerase recombinant kit (Life Technologies, Carlsbad, CA, United States). The 50 µl reaction mixtures consisted of 1 µl of 10 µM primers, 1 µl of 200 ng/µl genomic DNA of the different species or molecular grade water as a negative control (per PCR tube), 5 µl of iTaq Buffer 10×, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTPs (10 mM), 40.25 µl of molecular grade water and 0.25 µl of iTaq DNA Polymerase (5 U/µl). The PCR reactions were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States) with the following cycling protocol: 3 min at 95 °C, 30 cycles of 15 s at 95 °C, 50 °C, 55 °C or 60 °C for 30 s and 72 °C for 30 s; reactions were maintained at 4 °C. The annealing temperature was adjusted per primer to optimize the temperatures and concentration of the primers (57 ºC TOP I *S. aureus* and 58 ºC TOP II *C. albicans*). The PCR products were analyzed by agarose electrophoresis (1.5% agarose) under UV light using a gel-doc system (Bio-Rad Laboratories, Hercules, CA, United States) to check the product PCR size. All primers yielded a single band with the expected PCR product size (Table 2).

After determining the optimal concentration for each primer via qPCR (Alonso et al., 2018), conventional PCR reactions were performed for PCR product purification using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. These products were quantified in OD_{260nm} (Nanodrop DS-11 DeNovix, Wilmington, DE, United States) and verified for integrity in 1.5% agarose gel. The number of gene copies was calculated for each PCR product (Yin et al., 2001). Finally, these PCR products were subjected to serial dilutions to prepare the $2x10^{10}$ to $2x10^{2}$ copies/µL standard curves for qPCR reactions to determine gene expression (Alonso et al., 2018).

All qPCR experiments were performed according to the "MIQE Guidelines for qPCR" (Nolan et al., 2006). For qPCR reactions, the 1µL of +RT and -RT samples and specific primers at 10 µM were amplified and iQ SYBR Green Supermix # 1708882 (Bio-Rad Laboratories, Hercules, CA, United States), totaling a final reaction volume of 25 µL. The reactions were subjected to the following cycle in the CFX96 equipment (BioRad): step 1 (1X) 95ºC / 3 min; step 2 (35X) 95ºC / 15 sec, 58ºC / 30 sec, 68ºC / 15 sec, where data collection and analysis took place in real-time; step 3 (1X) 95 ° C / 1 min; step 4 (1X) 55 ° C / 1 min; step 5 (80X) 55ºC / 1 min, for the analysis of the melting curve. The annealing temperature was adjusted for each primer (Table 2). Each run was performed with the plate containing the standard curve and the cDNA samples from the different groups. The standard curves based on the PCR product were used for each primer as previously described (Yin et al., 2001). These curves resulted in a correlation coefficient of approximately 1, reaction efficiency of 95-105%, slope of about -3.3, and ideal melt curves. The amplification plot, standard curves, and melt curves were analyzed by the Bio-Rad CFX Manager™ Software (Bio-Rad Laboratories, Hercules, CA, United States). All samples were analyzed in duplicate. The data regarding the gene expression TOP II of *C. albicans* and TOP I of *S. aureus* from all groups were compared with the positive control group (cells without treatment).

Statistical analysis

Data obtained from all tests were subjected to statistical analysis using the SPSS Statistics software (IBM, USA). The assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Levene) of the data were accepted, and the one-way Analysis of Variance was applied. The Tukey's post-test was used for multiple comparisons (α 5%).

3. Results

Screening results: XTT analysis

The first step in this work was to assess metabolic mitochondrial activity of cells exposed to LED at different wavelengths, using the XTT assay. The blue LED results demonstrated a reduction in the metabolism of *C. albicans* compared to the control group with the application of doses of 100, 60, 50 and 37,5 J/cm^2 ($p \leq 0.0001$; Figure 1a). The other doses were similar to the negative control for the fungi ($p \geq 0.05$). For *S. aureus*, the greatest reduction in metabolism was also obtained with the higher doses: 100, 60, and 50 J/cm^2 ($p \leq 0.0001$). The doses of 37,5; 15 and 8 J/cm^2 also promoted a significant reduction in bacterial metabolism ($p \leq 0.0001$), when compared with the positive control but in lower extent. (Figure 1b). Based on these results, the blue LED doses selected for the subsequent tests were 15 and 50 J/cm^2.

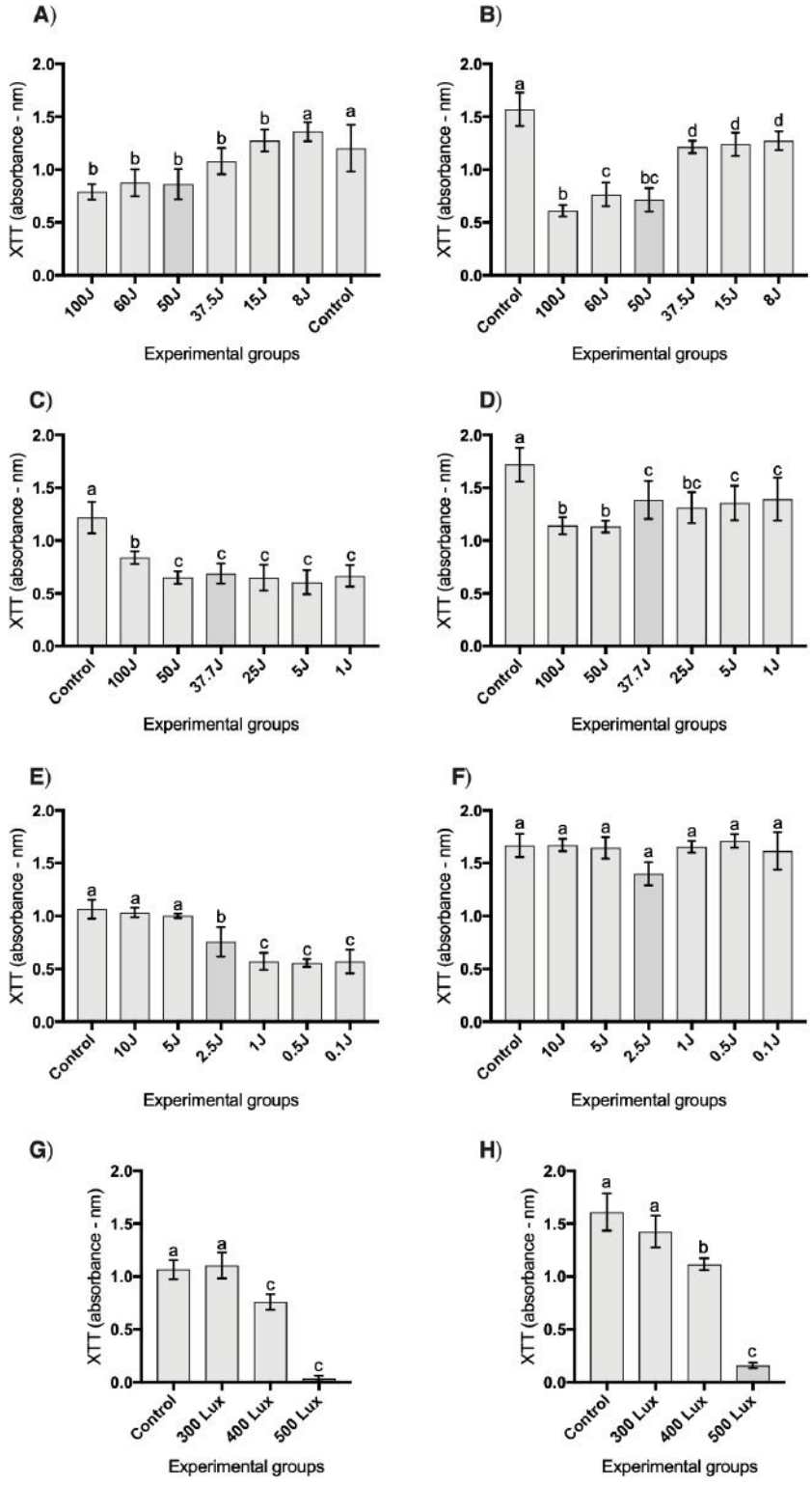
The red LED application significantly reduced the metabolism of *C. albicans* suspensions, regardless of the light dose used (Figure 1c). All doses of red LED also caused a significant reduction in *S. aureus* metabolism. Doses of 100, 50 and 25 J/cm^2 ($p \leq 0.0001$, when compared with the control) were statistically similar to each other and showed the highest reduction values, where the other doses evaluated showed a slight reduction, with values statistically different from the negative control ($p \leq 0.05$; Figure 1d). Thus, doses of 5 and 50 J/cm^2 were selected for the other evaluations.

For the yellow LED, doses of 2.5, 1.0, 0.5, and 0.1 J/cm^2 significantly reduced the metabolism of *C. albicans* compared to the negative control ($p \leq 0.0001$; Figure 1e). The light doses of 10 and 5 J/cm^2 did not affect the metabolism of the fungus, showing statistically similar activities regarding the negative control ($p = 0.984$ and $p = 0.659$, respectively; Figure 1e). Regarding *S. aureus*, none of the tested yellow light doses affected bacterial metabolism compared to the control group ($p \geq 0.05$; Figure 1f). Thus, doses of 0.1 and 10 J/cm^2 were selected to carry out the other experiments.

The results showed that the white LED at 500 lux and 400 lux intensity caused a significant reduction in the metabolism of *C. albicans* and *S. aureus*, which was different from the negative control for both microorganisms ($p \leq 0.0001$; Figure 1g and 1h, respectively). On the other hand, the intensity of 300 Lux did not affect the metabolism of the microorganisms evaluated ($p = 0.724$ and $p = 0.07$, respectively). Thus, the doses of 300 and 500 lux were evaluated in the next tests.

It is important to mention that after XTT assay, 2 doses of light of each LED were chosen to be evaluated by the other tests: a dose of light that caused great reduction and another one that did not alter the cell metabolism.

Figure 1- Cellular metabolism assessed with the XTT assay of *C. albicans* (A; C; E; G) and *S. aureus* (B; D; F; H) obtained after lighting with different doses of blue LED light (A; B), red LED (C; D), yellow LED (E; F) and white LED (G; H). Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$; $n = 10$)

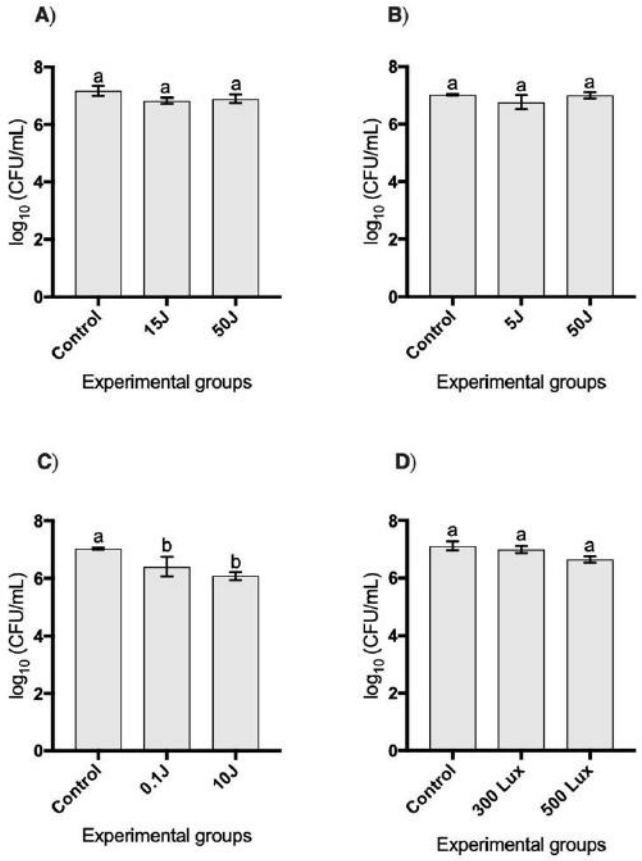


Source: Authors.

Cell viability by CFU/mL

Regarding cell viability, only the yellow LED at doses of 0.1 and 10 J/cm^2 promoted a significant reduction in *C. albicans*, compared to the negative control (p = 0.025 and p = 0.015, respectively), which were equivalent to 0.5 and 0.6 $\log_{10}$, respectively (Figure 2c). The blue, red, and white LED, in the evaluated doses, showed statistically similar behavior to that of the control group for *C. albicans* (p = 1.0; Figure 2a, 2b, and 2d, respectively).

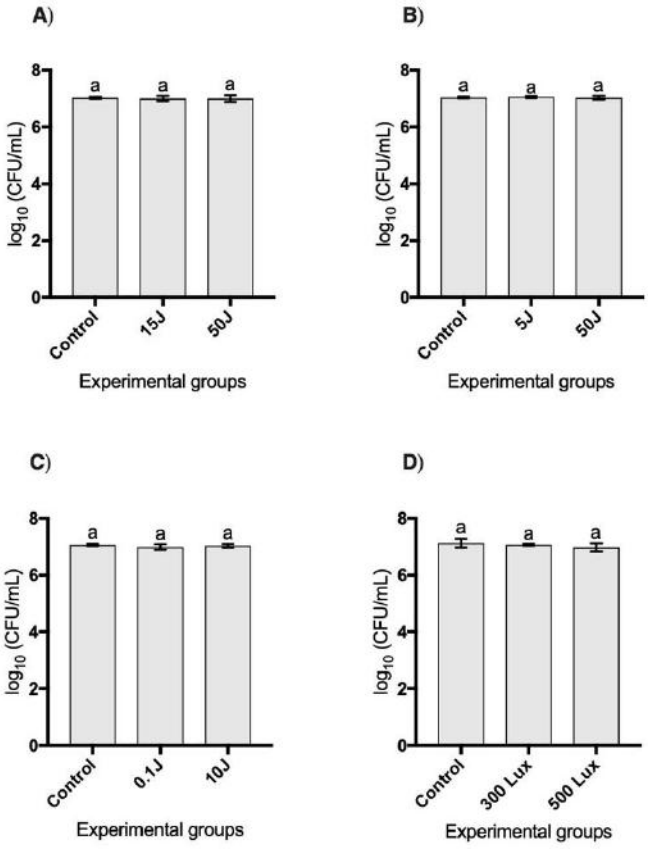
Figure 2 - Cell viability in $\log_{10}$ (CFU/mL) of *C. albicans* obtained after illumination with blue LED light (15 and 50 J/cm^2) (A), red LED (5 and 50 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). Different superscript letters denote statistical difference between columns (experimental groups) according to the Tukey`s post-test (p <0.05; n = 10).



Source: Authors.

However, for *S. aureus*, none of the evaluated wavelengths reduced cell viability, which was similar to the control at all doses tested (p> 0.05) (Figure 3a, 3b, 3c, and 3d).

Figure 3- Mean values and standard deviation of cell viability in log_{10} (CFU/mL) of *S. aureus* obtained after illumination with blue LED light (15 and 50 J/cm^2) (A), red LED (5 and 50 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). Control- cells without treatment. Different superscript letters denote statistical difference between columns (experimental groups) according to the Tukey`s post-test ($p < 0.05$) (n = 10).



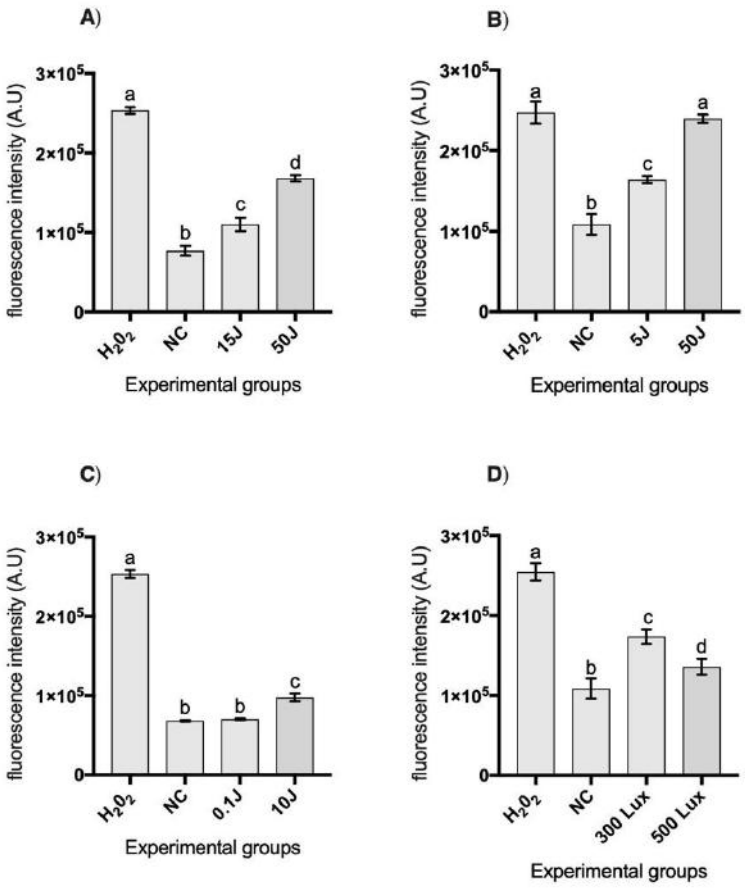
Source: Authors.

Analysis of ROS production

The application of blue and red LED significantly increased the production of intracellular ROS in *C. albicans* (Figs. 4a and 4b) and *S. aureus* (Figure 5a and 5b) at both evaluated light doses (15 and 50 J/cm^2) when compared to the control. Interestingly, ROS production was dependent on the dose in *C. albicans* (Figs. 4a and 4b), and in *S. aureus* with blue LED (Figure 5a). The yellow LED caused a significant increase of intracellular ROS production at a light dose of 10 J/cm^2 for both microorganisms (Figs. 4c and 5c) when compared with the group treated with H_2O_2 ($p \leq 0.0001$). The dose of 0.1 J/cm^2 demonstrated similar values to the negative control for both microorganisms ($p \leq 0.05$).

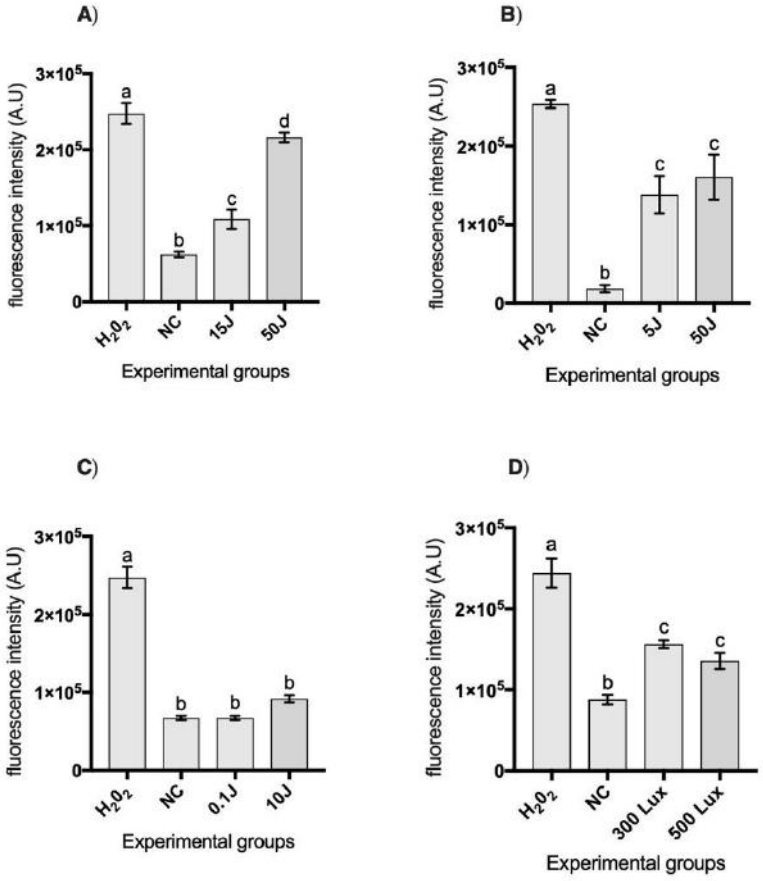
The white LED increased the production of intracellular ROS in *C. albicans* (Figure 4d), and *S. aureus* (Figure 5d) at the two light intensities used (300 and 500 lux). However, cells of *C. albicans* illuminated with 300 lux produced a larger amount of ROS compared to the 500 lux dose and the negative control ($p \leq 0.0001$; Figure 4d), while for *S. aureus*, no significant differences were observed for both LED doses ($p > 0.05$; Figure 5d).

Figure 4- ROS production measured by fluorescence intensity the redox-sensitive fluorochrome dichlorofluorescein diacetate of *C. albicans* obtained after illumination with blue LED light (15 and 50 J/cm^2) (A), red LED (5 and 50 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). The negative control (NC) was prepared with untreated cells with fluorochrome and the positive control (H_2O_2) with 10 mM H_2O_2 for 20 min. Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$; n = 10; AU means arbitrary units)



Source: Authors.

Figure 5 - Mean values and standard deviation of ROS production measured by fluorescence intensity (AU - arbitrary units) of *S. aureus* obtained after illumination with blue LED light (15 and 50 J/cm^2) (A), red LED (5 and 50 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). Hydrogen peroxide (H_20_2). Negative control (NC) - treated cells with fluorochromism. Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$) ($n = 10$).

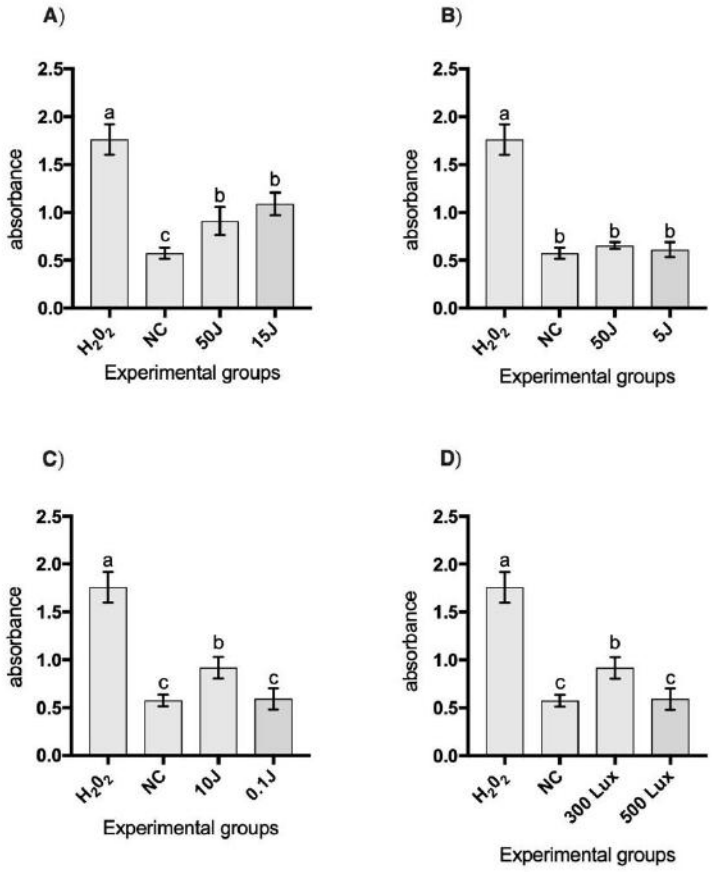


Source: Authors.

Analysis of lipid peroxidation

The illumination with blue LED (15 and 50 J/cm^2; Figure 6a) and white LED (300 lux; Figure 6d) caused lipid oxidation in the cells of *C. albicans* compared to the negative control ($p \leq 0.0001$) and significantly lower than the group treated with H_2O_2 ($p \leq 0.0001$). Lighting with 10 J/cm^2 of yellow light caused a significant increase in peroxidation in *C. albicans*, which was different from the negative control ($p \leq 0.0001$; Figure 6c) and the control treated with H_2O_2 ($p \leq 0.0001$; Figure 6c). On the other hand, red LED lighting (5 and 50 J/cm^2; Figure 6b) did not promote lipid oxidation in *C. albicans* cells when compared to the negative control ($p = 0.967$ and $p = 0.768$, respectively).

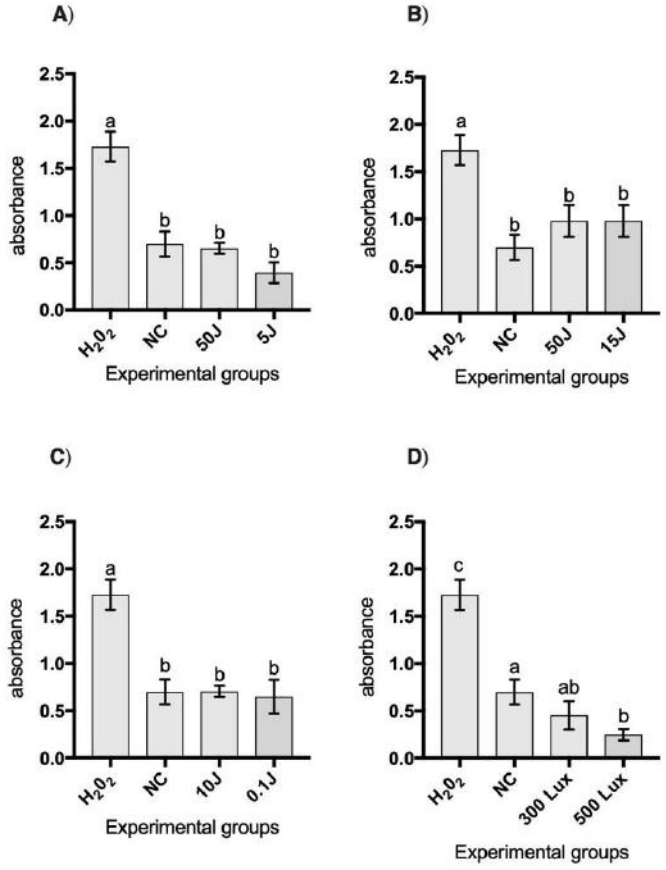
Figure 6 - Lipid peroxidation measured with the FOX method of *C. albicans* obtained after illumination with blue LED (50 and 15 J/cm^2) (A), red LED (50 and 5 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). The negative control (NC) was prepared with untreated cells with fluorochrome and the positive control (H_2O_2) with 10 mM H_2O_2 for 20 min. Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey`s post-test ($p < 0.05$) ($n = 10$).



Source: Authors.

For *S. aureus*, LED lighting, regardless of the applied wavelength, did not cause lipid peroxidation, presenting a similar behavior to the control group ($p > 0.05$) (Figure 7).

Figure 7 - Mean values and standard deviation of lipid peroxidation measured by the absorbance (nm) of *S. aureus* obtained after illumination with blue LED (50 and 15 J/cm^2) (A), red LED (50 and 5 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). The negative control (NC) was prepared with untreated cells with fluorochrome and the positive control (H_2O_2) with 10 mM H_2O_2 for 20 min. Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$) (n = 10).

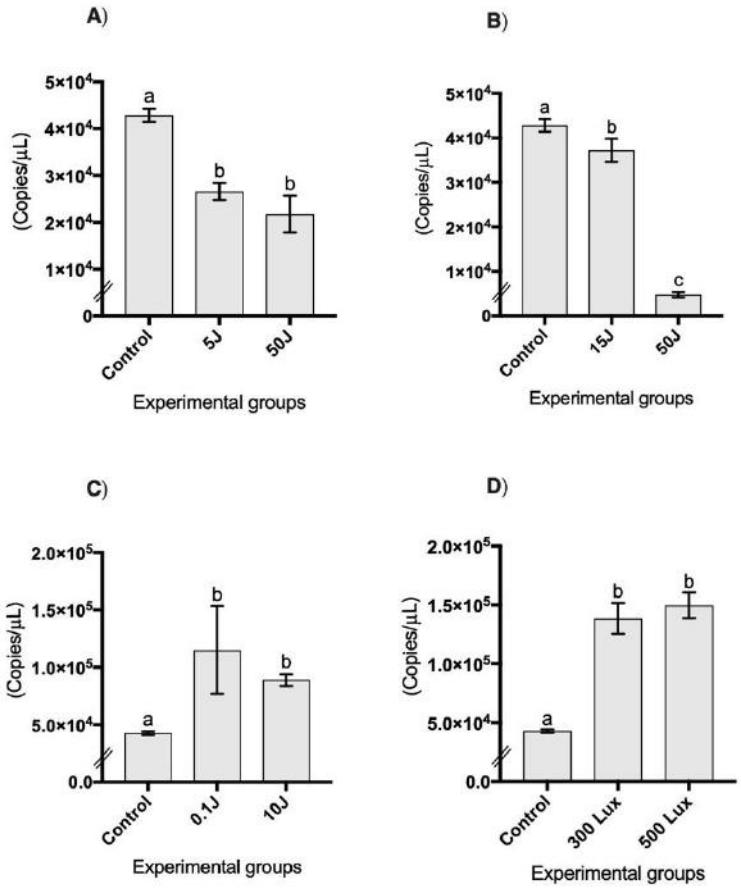


Source: Authors.

Topoisomerase enzyme expression analysis

The application of the two doses of red light (5 and 50 J/cm^2) significantly reduced the TOP II expression of *C. albicans* compared to the control ($p \leq 0.0001$; Figure 8b), as well as the application of blue light ($p \leq 0.0001$; Figure 8a), where the highest dose (50 J/cm^2) caused the greatest reduction in gene expression. Regarding yellow (0.1 and 10 J/cm^2) and white (300 and 500 lux) LED lights; all doses increased the expression of *C. albicans* TOP II significantly compared to the negative control ($p \leq 0.0001$; Figs. 8c and 8d).

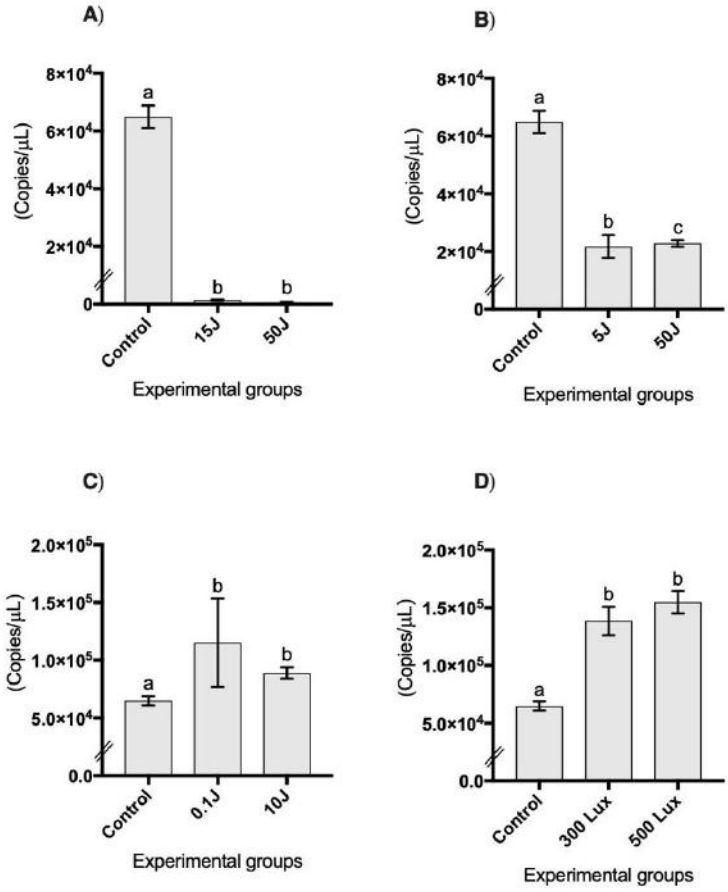
Figure 8 - Mean values and standard deviation of the gene expression of the TOP 2 enzyme (Copies/µL) by RT-qPCR of *C. albicans* obtained after illumination with blue LED light (15 and 50 J/cm^2) (A), red LED light (5 and 50 J/cm^2) (B), yellow LED light (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$) (n = 8).



Source: Authors.

The application of 5 and 50 J/cm^2 of red LED decreased the expression of the TOP I enzyme of *S. aureus*, compared to the negative control ($p \leq 0.0001$ for both light doses; Figure 9b). Regarding the blue LED, the application of 15 and 50 J/cm^2 promoted high reduction in the *S. aureus* TOP I expression compared to the control ($p \leq 0.0001$; Figure 9a). On the other hand, the application of yellow and white LED (at the different doses evaluated) upregulated the expression of the enzyme TOP I of *S. aureus*, in comparison with the negative control ($p \leq 0.0001$; Figs. 9c and 9d).

Figure 9- Mean values and standard deviation of the gene expression of the TOP 1 enzyme (Copies / µL) by RT-qPCR of *S. aureus* obtained after illumination blue LED (15 and 50 J/cm^2) (A), red LED (5 and 50 J/cm^2) (B), yellow LED (0.1 and 10 J/cm^2) (C) and white LED (300 and 500 lux) (D). Different superscript letters denote the statistical difference between columns (experimental groups) according to Tukey´s post-test ($p < 0.05$) (n = 8).



Source: Authors.

4. Discussion

The present study evaluated the action of different wavelengths of LED (blue, red, white, and yellow) on metabolic mitochondrial activity, cell viability, lipid peroxidation, ROS production, and gene expression of Topoisomerase I and II in suspensions of *S. aureus* and *C. albicans*, respectively. The blue and red LED promoted a reduction in metabolic activity from the dose of 50 J/cm^2 for *C. albicans* and *S. aureus.* The white LED at 500 lux caused a significant reduction in the mitochondrial activity of *C. albicans* and *S. aureus*, which was approximately 80 and 73%, respectively. In contrast, the yellow LED promoted a reduction in the metabolism of only *C. albicans* (~ 48% with 0.1 J/cm^2).

The mechanism of action of phototherapy is due to the photo-excitation of endogenous porphyrin molecules, a process that culminates in the ROS production, including singlet oxygen (O_2), superoxide anion (O_2^-), hydrogen peroxide groups (H_2O_2), and hydroxyl (OH) (Devasagayam et al., 2003). These molecules are chemically reactive to cell constituents. Their overproduction becomes toxic to cells, altering the redox balance, damaging cell structures through the oxidation of macromolecules, such as proteins, lipids, nucleic acids, NADH/NADPH, and soluble thiols (Devasagayam et al., 2003). The light absorbed at different wavelengths inhibits bacterial growth by two main mechanisms: photochemical and photothermal (Feuerstein, 2012). The photochemical mechanism is based on the formation of ROS. In the absence of exogenous

photosensitizers, visible light mainly affects porphyrin-containing black bacteria, such as *P. gingivalis* (Feuerstein et al., 2005). The present study results corroborate those in the literature because the intracellular ROS production was observed in practically all tested LED light doses of all wavelengths, except for the dose of 0.1 J/cm^2 of yellow LED. Considering that ROS have an extremely short half-life, the application of 0.1 J/cm^2 of yellow light may not have been detected by the probe used here because it is a very low dose of light (20 seconds of application). The production of intracellular ROS was demonstrated in different microorganisms such as *C. albicans*, *Aggregatibacter actinomycetemcomitans*, *Staphylococcus epidermidis,* and *P. gingivalis* after phototherapy with blue LED [Carmello et al., 2015; Galbis-Martinez et al., 2012; Cieplik et al., 2014; O'Donoghue et al.,2016; Ramakrishnan et al., 2016; Yoshida et al., 2017).

In addition to intracellular ROS production, the results demonstrated the occurrence of lipid peroxidation in *C. albicans* after exposure to the yellow (10 J/cm^2), blue (15 and 50 J/cm^2), and white (300 and 500 lux) LEDs. In a previous study, lipid oxidation was observed in fungi (*Malassezia furfur, Malassezia sympodialis*, and *Malassezia globosa*) treated with the 392 (UV) and 415 nm wavelengths (blue) (Wi et al.,2012). A study evaluated the effect of different wavelengths of LED light (370–630 nm) on *Malassezia* species; the lipid peroxidation was increased by LED irradiation with 392.5 and 415 nm (Wi et al., 2012). Lipid peroxidation is an oxidative damage that affects cell membranes, lipoproteins, and other molecules and cellular structures that contain lipids under conditions of oxidative stress (Cheeseman, 1993). Cell membrane lipids most often represent oxidative attack substrates [Dai et al., 2012; Nawrot et al.,2011). Lipid peroxidation is a chain reaction that occurs when a free radical extracts an electron from a lipid, forming a fatty acid radical that reacts with other fatty acids, producing lipid peroxides and more fatty acid radicals. Once activated, the reaction continues autocatalytically; it has a progressive course that results in structural and functional changes in the substrate [Cuypers et al., 2010; Ognjanovic et al., 2008). Therefore, repeated cycles of lipid peroxidation can cause severe damage to cell membranes. Other products from damage caused by oxidative stress are reported, such as fat breakdown from lipid peroxidation (Pryor, 1991). In general, although the production of ROS and lipid peroxidation was observed after phototherapy with practically all the LEDs used, this production did not seem to be sufficient to reduce the viability of the evaluated fungal and bacterial cells.

In addition to the phototoxicity of LEDs being related to increased ROS production and lipid peroxidation, DNA damage in target cells has also been reported (Carmello et al., 2015). For this reason, the present study evaluated the effect of LEDs tested here on the gene expression of the Topoisomerase enzymes for *C. albicans* and *S. aureus*. In the present study, both the application of 15 and 50 J/cm^2 of blue light and the application of 5 and 50 J/cm^2 of red light promoted a reduction in the expression of TOP II e I for *C. albicans* and *S aureus*, respectively. Topoisomerases enzymes play essential roles in promoting DNA replication and transcription, in addition to stabilizing transient DNA breaks through the formation of covalent tyrosyl phosphate intermediates (Silver,2011). The mechanism of action of these enzymes, which are essential in controlling the microbial topology of DNA, involves the cleavage of DNA and the passage of a second double strand of DNA, followed by the rewiring of the cleaved DNA [Corbett & Berger,2004). Thus, one of the possible mechanisms of action of red and blue LED lights for microbial inactivation is the decrease in the expression of genes that encode Topoisomerase enzymes, which are crucial for cell viability since their suppression reduces the repair of damaged DNA after phototherapy. On the other hand, the white and yellow LEDs caused an increase in the expression of the genes encoding these enzymes in both microorganisms. These findings associated to the reduction in the metabolic activity of the cells allows to suggest that the white and yellow LED may inactivate the microorganisms by other mechanisms and not for genotoxicity. Taken together, these findings highlight the need to carefully choose the wavelength in phototherapy, since microbial inactivation efficacy can be highly dependent on the type of light.

Here, the blue (455 nm), red (660 nm), and white LED, in the doses evaluated, did not promote a reduction in the viability of *C. albicans* or *S. aureus*. In contrast, the yellow LED (590 nm) caused a statistically significant reduction in *C.*

albicans viability in the light doses of 10 and 0.1 J/cm^2 but did not affect the *S. aureus* viability. Each wavelength of LED has different depths of penetration into cells and acts on different intracellular targets (de Freitas & Hamblin, 2016), which may justify the results found here for the yellow LED versus the others LED wavelengths. The antimicrobial activity of blue LED light has been described however; different wavelengths were used within the light absorption spectrum and different light doses. The doses ranging between 118 and 2214 J/cm^2 at 405 nm demonstrated complete inactivation (> 4 $\log_{10}$ CFU) of suspensions of clinical isolates of *S. aureus*, *Escherichia coli,* and *C. albicans* (Gupta et al., 2015). A ~ 5 $\log_{10}$ reduction in cell viability of *C. albicans* and *Saccharomyces cerevisiae* suspensions was also observed after exposure to 576 J/cm^2 and 288 J/cm^2 at 405 nm, respectively (Murdoch et al., 2013). In adittion, a reduction of more than 4 $\log_{10}$ CFU was observed in suspensions of *C. albicans* after exposure to 70 J/cm^2 at 415 nm (Zhang et al., 2016). In the same way, as observed for *C. albicans*, other studies have shown susceptibility of suspensions of *S. aureus* to phototherapy mediated by the blue LED. A reduction of ~ 5 $\log_{10}$ in bacterial strains causing hospital infections, including *S. aureus*, was found after exposures to 54 to 108 J/cm^2 at 400 nm (Halstead et al.,2016). Similarly, inactivation of more than 5 $\log_{10}$ of *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. pneumoniae* was observed after exposure to 133 J/cm^2, at 405 nm (Barneck et al., 2016). The difference observed of the results from the literature when compared to our results (Figures 2 and 3) can be attributed to the different doses of light used. Another aspect that may be considered is the different wavelengths within the light absorption spectrum, strains used and cultivation procedures used, since the microbial cell growth phase may also be a factor that produce distinct response to the tested therapies.

The doses (5 and 50 J/cm^2) of red LED light at 660 nm did not significantly reduce the cell viability in the evaluated microorganisms. These findings are in line with the few studies found on phototherapy with red LED/Laser light, which suggest that lights at this wavelength (660 nm) do not have antimicrobial effects [Feuerstein et al., 2004; Nussbaum et al., 2002]. Likewise, the two intensities of white LED light (300 and 500 lux) evaluated did not affect the cell viability of both microorganisms. There are no previous reports on the effect of a white LED on the bacterium and fungus tested.

5. Conclusion

In summary, in this work we show that the mechanisms of action of white LEDs and at blue (455 nm), red (660 nm) and yellow (590 nm) wavelengths seem to be related to the production of ROS, lipid peroxidation, and DNA damage. This work constitutes a first step towards standardized lighting protocols that can be developed, and to understand the mechanisms of action of the various wavelengths of the LEDs on microbial cells before clinical application.

Acknowledgments

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