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Assessment of high-power LED in the adhesion of wild *Pseudomonas aeruginosa* isolates.

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ABSTRACT

The use of high-power LED radiation in the visible light length serves as a suitable alternative to traditional ways of combating microbial growth, reducing occupational risk and environmental impact. This study aimed to verify the adhesion rate of three wild isolates of *P. aeruginosa* and a standard strain, under high power LED radiation. The organisms were exposed to LED radiation at 460, 580 and 637 nm for 24 hours. The biofilm formed was then quantified by the violet crystal test and the adhesion rate was calculated by the difference between the absorbance (λ= 590 nm) of the treated and the control material. Cell viability and biofilm formation were observed as a function of the applied wavelength under all conditions tested. The adhesion rate varied between 50-95% and in general, the highest and lowest changes in the biofilm formation occurred at λ= 637 and 580 nm, respectively, suggesting that the high power LED light energy alone was not enough to ensure inactivation of *P. aeruginosa*.

Keywords: biofilms, photostimulation, visible light.

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INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative rod commonly found in soil, and producer of fluorescent pigments. [1] In addition, the bacterium exhibits the ability to organize itself into biofilms, [2] and this organizational microbial lifestyle provides protection against various toxic agents, allowing mutations [3] Additionally, *P. aeruginosa* is considered an opportunistic pathogen in humans, causing concern because of its multidrug resistance, leading to nosocomial infections with a high incidence of morbidity and mortality. [4]

The control of microbial growth is the way to avoid contamination and spread of microorganisms in environments, eliminating or reducing them to a controllable number. [5] Control techniques vary in contact time, involving physical and/or chemical agents. [6] These disinfection/sterilization protocols, when performed incorrectly, may affect the microbial balance in the natural environment. And when these are discarded, incorrectly processed materials continue to promote contact between microorganisms and antimicrobial agents, which can lead to resistance. Given this, new disinfection strategies need to be tested, [7] for example ozonation, application of ultraviolet (UVC) and ultrasonic vibration. [8-9] These techniques are used to reduce the impacts on health and the environment, caused by traditional techniques for controlling microbial growth. [10]

LED radiation is a clean and powerful source of energy and may be seen as an alternative to the harmful techniques mentioned above in the control of microbial growth. [11] LED has certain benefits, making it better than other methods using light energy, for example the mercury gas lamp, which in addition to the short durability, can cause contamination if the material is spilled. [12-13]

LED can be classified according to its frequency, being considered high when greater than 43Hz. [14] High-power LED is the most widely technique used to control microbial growth. [15] The action of high-power LED on *P. aeruginosa* occurs at the molecular level, promoting changes linked to inactivation or decreased expression of the genes responsible for coordinating responses of cell population density. Some of these may be effects on cell proliferation and maintenance, cell behaviour, horizontal transfer of genes and interactions with the environment. [16] The effectiveness of high-power LED seems to act most effectively on cell behaviour, since it can also disrupt biofilm formation. [17]

Although different ways of controlling microbial growth are available today, many techniques involve occupational risk. [18] In addition, they may shorten the useful life of hospital equipment that undergoes decontamination [19], as well as damage the microbial balance of the natural environment. [20] Alternative ways of controlling microorganisms, which end or reduce these adverse effects, have become the target of scientific investigations. [21-22]

Identification of the possible damage that the high power LED can cause to the aggregation of *P. aeruginosa* is a way of understanding the basic effect of the emission of this light on the microbial cells, given that it comes from a more lasting and economical energy source, thus less harmful to the environment. The objective of our study was to verify the adhesion rate of *P. aeruginosa* under high power LED radiation at different wavelengths.

MATERIAL AND METHODS

Microbes

Three wild isolates of *Pseudomonas aeruginosa*, TGC01, TGC02 and TGC04 were used in the experiments. They were recovered from the soil taken from gas stations. [23] A standard strain of UFPEDA 416 was used for comparison proposes. All wild strains are registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number A6D0C2F.

LED

A 2m long LED strip (Nitrolux, RGB-Branca) was used. It was composed of 108 LED chips, with 12V and 2.4 W/m voltage, 50/60 Hz and 5A, emitting 288 J/min..

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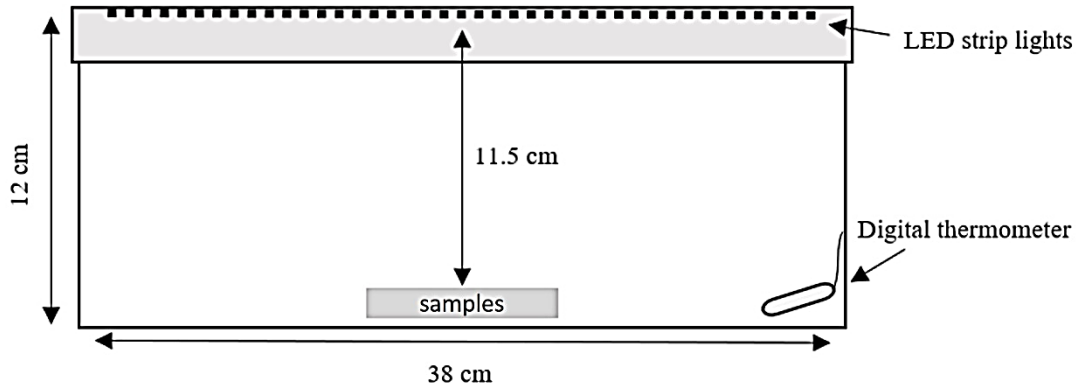
***In vitro* test of biofilm formation**

The *P. aeruginosa* isolates and the standard strain were incubated on nutrient agar for 24 hours at 30°C. Suspensions from the recent cultures, were prepared in 0.9% NaCl solution, and compared to the standard turbidity of the 0.5 tube on the MacFarland scale. Then, 100 µL of the *P. aeruginosa* suspension was added to 900 µL of nutrient broth in microtubes with a 1.5 mL capacity. The microtubes were sealed with coverslips and incubated in a box with the following dimensions: 28 cm wide x 38 cm long x 12 cm high. The microtubes were 11.5 cm from the light source. The light source was a 2m LED strip fixed on the box lid (Figure 1).

Fig. 1: Representation of the box used in the *in vitro* biofilm formation test.



The system was incubated at room temperature, monitored with a digital thermometer (Incoterm, 7665.02.0.00). Three wavelengths were used: 637 nm ($3.11x10^{-19}$ J, $4.71x10^{-14}$ Hz), 580 nm ($3.42x10^{-19}$ J, $5.17x10^{-14}$ Hz) and 460 nm ($4.31x10^{-19}$ J), $6.51x10^{-14}$ Hz). Afterwards, the violet crystal test was performed. [24] Initially, the supernatant in the microtubes was discarded and the walls were washed with running water, then dried for an hour. Then, 1000 µL of the violet crystal solution was added and after 20 minutes, the dye solution was discarded and the excess removed with running water. Then, the microtubes were filled with 1000 µL of absolute ethanol. The absorbance of the crystal violet-ethanol solution was measured on a spectrophotometer at λ= 590 nm (Quimis U2M).

The cell adhesion rate on the microtube walls was calculated using the formula [(ODC - ODT) ÷ ODC x 100], where ODC is the mean of the optical density of the control in nutrient broth and ODT, the mean of the optical density of the treatment with the LED. [25] Cell adhesion was classified as poor when <40%; moderate, when ≤40 and ≥80%; and strong, when >80%. [26] The tests were performed in triplicate and the test control was carried out under incubation under white LED radiation and in the dark. The temperature was monitored in all tests.

Statistical analysis

The analysis of data normality was performed using the Kolmogorov-Smirnov test. For the difference tests between groups, the ANOVA test was used, followed by the Tukey post-test.

RESULTS

All isolates formed biofilms under the conditions tested, indicating normal distribution (α= 0.05). Variations depended on the tested wavelength, compared with controls, under white light and in the dark (Table 1).

Table 1: Optical density obtained after LED radiation for 24 hours (SD=±0.2).

P. aeruginosa	Light energy			
	460nm	637nm	580nm	Luz branca
TGC01	0.695	0.814	0.297	0.380
TGC02	0.506	0.536	0.155	0.204
TGC04	0.465	0.455	0.486	0.489
UFPEDA 416	0.675	0.553	0.195	0.556

Optical density of growth medium = 0.057.
Control in the dark: TGC01 (0.318), TGC02 (0.245), TGC04 (0.283) and UFPEDA 416 (0.434)

The wavelength influenced the formation of biofilms, as well as the differences between the isolates depending on the treatment applied. The TGC01 and TGC02 isolates, λ= 460 and 637 nm, modulated the biofilm formation ($p>0.001$). In contrast, the effect of different wavelengths on the TGC04 isolate was indifferent, given that the same amount of biofilm was formed both under white light and in the dark ($p=0.045$). For the standard strain, formation of biofilm was affected only at λ= 460 nm ($p=0.02$), while at λ= 580 nm, it was reduced. Under the other conditions, the statistical analysis was not conclusive.

The analysis of the behaviour of both the isolates and the standard strain under the conditions tested indicated that there was a significant difference between them only at λ= 460 nm ($p= 0.022$). The TGC01 isolate produced the highest amount of biofilm, followed by TGC04, TGC02 and the standard strain. A difference in the TGC04 isolate was also observed at λ= 580 nm ($p=0.002$). The other isolates tested under the same conditions provided similar results, with no statistical difference (λ= 637 nm, $p=0.06$; white light, $p=0.22$). The results also indicate the presence of viable pioneer cells, i.e., the first planktonic cells that adhere to the substrate. This is based on the premise that the value of the measured optical density, in treatments with LED radiation and in the dark, may be above, close to, equal to or below a cutoff of the optical density. The cutoff calculation is based on the absorbance measurement of the medium used in the test. Three times this value sets the cutoff point, to make the adherence clearer to identify. The lower the cutoff value of the optical density, the weaker the adhesion, which is also indicative of the stimulating or inhibiting potential of radiation on the formation of the biofilm. In this test, the cutoff value was 0.189.

Table 2 summarizes the percentage of adherence given by statistical analysis for the formation of the biofilm in the tested *P. aeruginosa* isolates. The adhesion rate of the isolates and the standard strain varied between approximately 50 and 95%, ranging from moderate to strong, observing the greatest reduction at λ= 580nm.

Table 2: *P. aeruginosa* adherence rate after LED radiation for 24 hours (SD=±0.3)

P. aeruginosa	Light energy			
	460nm	580nm	637nm	Luz branca
TGC01	92.8	73.4	94.6	85.5
TGC02	90.,8	49.0	91.8	73.0
TGC04	89.0	83.7	90.3	88.8
UFPEDA 416	92.6	59.5	77.4	90.1

Control in the dark: TGC01 (82.3%), TGC02 (77.6%), TGC04 (80.6%) and UFPEDA 416 (87.3%). Moderate adhesion (40-80%), strong adhesion (> 80%)

DISCUSSION

Light energy plays a key role in microbial growth, serving as a source of energy and heat and assisting in biofilm adhesion and formation. [27] However, certain wavelengths may interfere with the development of microbial cells, which can suffer significant damage and consequently disturbances in the formation of biofilms. Based on this premise, light energy at different wavelengths needs to be used to control the growth of microbial cells. [28-29]

Photodynamic therapy employing LED energy is recognized as an efficient alternative method for controlling microbial growth in different environments. [30] The technique has as one of its main advantages: the fact that it is a source of clean energy. Thus use of photodynamic therapy can serve as a strategy to prevent the occurrence of bacterial resistance in detergents and antiseptics, as well as to minimize the environmental and occupational hazard impact caused by traditional techniques. [31] There are certain limitations to photodynamic therapy, however, especially when exposure to UV radiation is used. Recovery of affected microbial cells can occur through the photoreactivation process, which culminates in the repair of damaged DNA. [32].

Another limitation of photodynamic therapy concerns the use of the chemical substances used as photosensitizers, i.e., molecules which are absorbed by cells and stimulated by light, causing the formation of reactive oxygen species (ROS) in the intracellular environment, resulting in inactivation of physiological processes or the death of microorganisms. [33] It turns out that many of these substances are toxic and synthetic in origin, for example, safranin [34] and methylene blue. [35] These compounds are associated with important health and environmental hazards. [36] This further justifies closer evaluation of the use of high-power LED radiation, with a wavelength in the range of visible light, on the rate of adhesion of wild *P. aeruginosa* isolates without the use of photosensitizer.

Only the high-power LED radiation did not disturb either the biofilm formation of wild *P. aeruginosa* isolates or the standard strain under most of the conditions tested, including those using white light LED. On the other hand, significant differences between the isolates were observed in the formation of biofilms, reflected in the variation in adherence, identified as moderate to strong. The greatest adhesion disturbances were observed with TGC02 and UFPEDA 416 at λ= 580 nm. At this wavelength, only TGC04 exhibited high adhesion. A similar result occurred in a recent study, where *Staphylococcus carnosus* was exposed to λ= 500 nm and a subtle reduction in cell adhesion was observed, [37] approaching the value obtained in the control of the present study.

In addition, the highest temperature record in the tests was achieved with radiation at λ= 580 nm, 33.5ºC. Under this condition, cell activity tends to increase with accelerated metabolism, the concentration of toxic metabolites increases, reducing the doubling time. [38] This mechanism may be the cause of the reduction in adhesion at this wavelength, even when the reduction was classified as moderate or strong.

Surprisingly, the effects of radiation under λ= 460 nm resulted in the formation of robust biofilms, identified by the high percentage of adhesion. At this wavelength an opposite result is expected, since the wavelengths in the blue light range inhibit microorganisms. [39] The action of LED at λ= 406 nm effectively reduced the population of *Listeria monocytogenes* planktonic cells, also inhibiting biofilm formation in a cold shelf simulation. [40] The same bacteria in an experiment on the rate of cell growth in salmon fish exudate produced high inhibition under radiation at λ= 460 nm. [41] Both studies were conducted at low temperatures. Compared with the results of the present study, these studies reaffirm that temperature can play a crucial role in the use of LED energy.

The biofilm formed by the wild isolates of *P. aeruginosa* used in this study may have been favoured by the ambient temperature conditions. Temperature is an essential factor for microbial growth. *P. aeruginosa* in particular is known to grow under a significant range of temperatures, from cold to above 40°C. [42] The optimal temperature for growth temperature coincides with the range observed in this study. The temperature varied between 30.0 and 33.5°C and the energy emitted between 4.32 and $3.12x10^{-19}$ J. The highest temperature also coincided with the highest wavelength tested (λ= 580 nm). In addition, the internal heating of the box may have stimulated growth, mimicking a bacteriological incubator.

Similar to what had been observed at λ= 460 nm, when wild isolates were exposed to λ= 637 nm, robust biofilms were formed in all cases, revealing the possible stimulation effect of this wavelength. This result was similar to others reported in the literature. In the evaluation of biofilm formation treated with LED at λ= 642 nm, there was no inhibition of *Listeria monocytogenes*, *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* 0157:H7, or *Salmonella typhimurium* (Gram-negative). [43] In addition, another study verified growth control with LED radiation at λ= 620 nm for *Legionella rubrilucens* and λ= 698 nm for *Pseudomonas fluorescens* and *Staphylococcus epidermidis*. [44] In all cases, the growth was not interrupted, nor was the biofilm formation inactivated. The reason for this phenomenon may be due to the high energy released in the

form of heat, related to the wavelengths of the red light band. Under these conditions, microorganisms may have their metabolism accelerated or maintained, since the wavelength is not cytotoxic. [45]

LED radiation acts negatively on microbial growth through the generation of ROS, affecting the internal content of cells, as well as the permeability of the membrane, causing death. [46] In microbes, some intracellular substances can act to destabilize the antioxidant activity. The production of protoporphyrin IX and coproporphyrin by *Helicobacter pylori* is an example. The molecules act as endogenous photosensitizers. When stimulated by light, especially in the wavelength range of blue light, together with intracellular oxygen they can react with nearby molecules, transferring hydrogen atoms or electrons, causing ROS production. [47] Cell death may also occur from the production of singlet oxygen. [48].

However, the formation of biofilm by wild isolates and by the standard strain *P. aeruginosa*, under high-power LED radiation, may have occurred due to the photoprotection mechanisms attributed to the bacterium. Among these are the expression of important ROS inactivating enzymes, such as catalase, superoxide dismutase and peroxidases. Additionally, the synthesis of pyocyanin also participates in the phenomenon of protection against oxidative stresses. It is an important blue pigment, produced exclusively by 90-95% strains of the species [49]. This mechanism is associated with the concentration of intracellular phosphate and governs, among other characteristics, the resilience of the bacteria in extreme cases of stress, when ATP can be formed and allows the maintenance of cells in a hostile environment. [50-51] In addition, as a pigment, pyocyanin can absorb the energy released by the light source, preventing damage to the cell. [52] It is important to note that all *P. aeruginosa* specimens tested in this study were found to produce significant amounts of pyocyanin. [53-54]

Given this, future studies need to evaluate the parameters of temperature control and exposure time in more depth so that the effects on the adhesion disturbance are clearer. *P. aeruginosa* is a species that demands attention, due to its physiological and metabolic characteristics that make it ubiquitous, resilient and, in certain cases, an important opportunistic pathogen.

CONCLUSION

Under the experimental conditions chosen for this study, no inhibition of the adhesion of wild *P. aeruginosa*, nor of the standard strain UFPEDA 416 was observed. However, the rate of adhesion was variable, with influence exerted by the light energy emitted linked to the temperature. The results suggest that the high-power LED light energy by itself is not enough to guarantee efficiency in the inactivation of biofilm formation. This light source can be used, however, to increase the biomass of *P. aeruginosa* in situations that demand its potential metabolic process, for example in bioremediation. Future studies by our group intend to investigate this application.

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