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ORIGINAL ARTICLE

Red and infrared laser therapy inhibits in vitro growth of major bacterial species that commonly colonize skin ulcers

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Abstract Low-level laser therapy (LLLT) is used in chronic wounds due to its healing effects. However, bacterial species may colonize these wounds and the optimal parameters for effective bacterial inhibition are not clear. The aim of this study was to analyze the effect of LLLT on bacterial growth in vitro. Bacterial strains including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were suspended in saline solution at a concentration of 10^3 cells/ml and exposed to laser irradiation at wavelengths of 660, 830, and 904 nm at fluences of 0 (control), 3, 6, 12, 18, and 24 J/cm^2. An aliquot of the irradiated suspension was spread on the surface of petri plates and incubated at 37 °C for quantification of colony-forming unit after 24, 48, and 72 h. Laser irradiation inhibited the growth of *S. aureus* at all wavelengths and fluences higher than 12 J/cm^2, showing a strong correlation between increase in fluence and bacterial inhibition. However, for *P. aeruginosa*, LLLT inhibited growth at all wavelengths only at a fluence of 24 J/cm^2. *E. coli* had similar growth inhibition at a wavelength of 830 nm at fluences of 3, 6, 12, and 24 J/cm^2. At wavelengths of 660 and 904 nm, growth inhibition was only observed at fluences of 12 and 18 J/cm^2, respectively. LLLT inhibited bacterial growth at all wavelengths, for a maximum of 72 h after irradiation, indicating a correlation between bacterial species, fluence, and wavelength.

Keywords Low-level laser therapy · Skin ulcers · *Staphylococcus aureus* · *Escherichia coli* · *Pseudomonas aeruginosa*

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Introduction

Bacterial species can be detected in almost all chronic wounds. However, from this initial colonization, more serious problems can occur such as infections or even sepsis [1]. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* are among the main bacterial species that commonly colonize skin ulcers [2–4]. In a study conducted by Korber et al. [1], 107 ulcers were investigated and 191 pathogenic bacteria were identified, with 55.1 % of ulcers colonized by *S. aureus*, followed by *P. aeruginosa* (33.6 %) and *E. coli* (7.5 %). In a recent study, colonization by *E. coli* was detected in 116 patients with diabetic ulcers, and in 35.71 % of these, 75 % were resistant to ampicillin [5].

In developing countries, it is estimated that 1–2 % of the population will develop chronic ulcers during their lifetime. A North American estimate reports that the cost of treating only one chronic ulcer is approximately US$8000 per year, increasing to US$17,000 when it is infected. Worldwide, the costs generated by care of chronic ulcers are approximately US$13–15 billion per year [6].

Low-level laser therapy (LLLT) has been used as an adjuvant therapeutic approach in the healing process. Fulop et al. [7] conducted a meta-analysis of studies published between 2000 and 2007 and found that the use of phototherapy had positive results on tissue repair. Additionally, Peplow et al. [8], in a review of studies from 2002 to 2009, found that the photobiomodulator effect of LLLT helped stimulate the proliferation of human and animal cells in vitro. Important for healing, Nussbaum et al. [9] reported that LLLT also played an important role in bacterial inhibition. They demonstrated varying biomodulator effects using different wavelengths, fluences, and bacterial species and found that the results were dependent on wavelength, bacterial species, time of exposure to irradiation, and irradiation fluence. However, most studies that investigated the irradiation of bacterial cultures were conducted using different methods [9–20], which can affect the absorption of irradiation.

In the literature, few studies have used different wavelengths of LLLT, with different fluences and time, to analyze the growth inhibition of major bacterial species that commonly colonize skin ulcers. Therefore, the aim of this study was to analyze the effect of LLLT in the red and infrared light spectra on *S. aureus*, *E. coli*, and *P. aeruginosa* growth in vitro.

Material and methods

Cell culture

S. aureus ATCC 25923 (gram-positive), *P. aeruginosa* ATCC 27853 (gram-negative), and *E. coli* ATCC 25922 (gram-negative) were purchased from ATCC. Bacterial cultures were maintained in Mueller-Hinton culture medium (BD®, New Jersey, PA, USA), replated, and incubated for 24 h at a temperature of 37 °C. Cells were then suspended in saline solution (0.9 % NaCl), then serial dilutions were carried out which were spectrophotometrically monitored using a Densicheck™ Plus spectrophotometer (bioMérieux®, Durham, NC, USA). Turbidity remained between 0.5 and 0.63, corresponding to a concentration of 1.5×10^8 colony-forming units (CFU)/ml on the McFarland scale. The cells were then serially diluted to a concentration of 1.5×10^3 CFU/ml. A 300-μL aliquot of this suspension was then transferred to individual wells of a microtiter plate for irradiation.

Irradiation

Bacterial suspensions were irradiated with a laserpulse (Ibramed®, Amparo, SP, Brazil) with diodes at wavelengths of 660 nm (red), 830 nm (infrared), and 904 nm (infrared) (Table 1). After irradiation, 100 μL of this suspension was transferred and spread over the surface of solid Mueller-Hinton culture medium in petri dishes (90 × 15 mm) and incubated at 37 °C for 24, 48, and 72 h, at which points the number of CFUs was determined using ImageJ 1.45® software (http://rsb.info.nih.gov/ij). All experimental procedures were performed in a sterile laminar flow hood. Each experiment was conducted in triplicate and repeated three times on different days, totaling 486 samples (3 wavelengths × 6 intensities × 3 series × 3 repetitions × 3 bacterial species). Approximately 20 % of samples were repeated due to contamination. Irradiation was conducted in a room with controlled temperature (23 ± 2 °C). The temperature of samples was monitored at different wavelengths and fluences (Table 2). The temperatures of two samples were measured in triplicate at each fluence and wavelength, using an infrared thermometer (Incoterm®, Porto Alegre, RS, Brazil).

Table 1 Irradiation parameters [units]

Center wavelength [nm]	660	830	904
Operating mode	Continuous	Continuous	Pulsed
Frequency [Hz]	–	–	9500
Pulse on duration [s]	–	–	60×10^{-9}
Duty cycle [%]	–	–	0.1
Peak radiant power [mW]	30	30	70×10^3
Average radiant power [mW]	30	30	40
Aperture diameter [cm]	0.16	0.36	0.48
Beam spot size at target [cm^2]	0.02	0.101	0.1808
Beam shape	Gaussian	Gaussian	Gaussian
Beam divergence [°]	22°	22°	11° × 25°

Energy density (fluence) of irradiation was calculated based on the formula below:

$$\mathbf{ED} = \mathbf{P} \times \mathbf{T}/\mathbf{A}$$

where:

ED energy density (J/cm^2)
P average power (W)
T irradiation time (s)
A irradiation area (cm^2)

The energy density presented on the LLLT considered the diode's irradiation area as a parameter for calculation. Because of the use of three separate diodes, with different areas and powers (Table 1), we made the decision to use the area of 1 cm^2 corresponding to the area of individual wells of the microtiter plate that contained the bacterial samples in suspension; this ensured that energy density and radiated energy were equal for all diodes. Thus, to irradiate the bacterial species, fluences of 0 (control), 3, 6, 12, 18, and 24 J/cm^2 were used for 0, 100, 200, 400, 600, and 800 s, respectively, at wavelengths of 660 and 830 nm. At 904 nm, the irradiation times were 0, 75, 150, 300, 450, and 600 s, respectively, at the same fluences. Control wells were not irradiated and were in

Table 2 Average values (standard deviation) of the temperature in degree Celsius

Wavelengths (nm)	Fluences (J/cm^2)						
	0	3	6	12	18	24	General average
660	24.93 (0.111)	24.82 (0.121)	24.92 (0.107)	24.88 (0.090)	24.90 (0.129)	24.92 (0.107)	24.89 (0.118)
830	24.97 (0.094)	24.85 (0.138)	24.93 (0.094)	24.92 (0.090)	24.92 (0.134)	24.90 (0.115)	24.91 (0.118)
904	24.95 (0.096)	24.83 (0.125)	24.97 (0.094)	24.92 (0.090)	24.93 (0.125)	24.95 (0.096)	24.93 (0.114)

ambient light. All irradiations occurred directly, punctually, and perpendicularly to the plate, and the emitter was fixed with the aid of a support, at a distance of 2 mm above the plate, over the area to be irradiated.

Measurement of diodes

The equipment was checked at the beginning and end of the experiment using a powermeter Field Max Top with PM3 sensor—0.5 mW to 2 W broadband sensor (RoHS) (Coherent®, Staunton, VA, USA) according to the method by Guirro and Weis [21]. Prior to beginning the irradiation experiments, we performed experiments to ensure uniformity of laser irradiation on bacteria in suspension. To do this, an experiment was conducted to analyze laser transmission in saline solution containing the bacterial species under study (at the same concentration and volume as our irradiation experiments). For this purpose, the bacterial sample was positioned between the diode and the powermeter and irradiated in a colorless solution (saline solution) to quantify the influence of the culture medium on light beam transmission. The results from this experiment showed that the light beam was slightly attenuated (Table 3).

Statistical analysis

Initially, the Shapiro-Wilk normality test was applied. Data on the number of CFU were analyzed using Kruskal-Wallis and post hoc Dunn's tests. The diodes' power, transmissivity, and temperature were analyzed using repeated measures ANOVA and post hoc Tukey's test. The correlation between inhibition and fluence of irradiation was analyzed using Spearman's correlation test. All tests were performed with the significance level of 5 % ($p < 0.05$) using SPSS version 17.0 software (IBM®, Chicago, IL, USA).

Results

Compared to the control, laser irradiation inhibited the growth of *S. aureus* at fluences higher than 12 J/cm^2, regardless of the wavelength used (Fig. 1; Table 4). Moreover, a high negative correlation between fluence and bacterial inhibition was observed (Table 5). When fluences were compared, the results showed that a fluence of 24 J/cm^2 led to greater inhibition than 3 J/cm^2 at all wavelengths. When different wavelengths were compared at a fluence of 24 J/cm^2, irradiation with red light (660 nm) led to greater inhibition of *S. aureus* than with infrared light (830 nm) (Table 4).

P. aeruginosa showed inhibition at 660 nm at fluences of 18 and 24 J/cm^2 at all time points, with the highest inhibition observed at 24 J/cm^2. At 830 nm, significant inhibition could be observed at fluences greater than 6 J/cm^2. At 904 nm, inhibition could only be observed at 24 J/cm^2 (Fig. 2; Table 4). Although different diodes and fluences led to varying degrees of inhibition, there was a negative correlation between bacterial inhibition and fluence at all wavelengths (Table 5).

As for *E. coli*, we showed that at a wavelength of 660 nm, inhibition could only be observed at 12 J/cm^2. At a wavelength of 830 nm, inhibition could be observed at 3, 6, 12, and 24 J/cm^2. At 904 nm, inhibition could be observed only at a fluence of 18 J/cm^2; however, at 3 J/cm^2, inhibition varied at 24 and 72 h after irradiation. Greater inhibition was observed at a wavelength of 830 than 660 nm at fluences of 3, 6, and 24 J/cm^2, as well as 904 nm at fluences of 18 and 24 J/cm^2 (Fig. 3; Table 4). For *E. coli*, a low negative correlation was observed between fluence and number of CFUs, for all wavelengths.

Discussion

As previously mentioned, there is no consensus regarding the most appropriate wavelength and fluence to use to inhibit bacterial growth. One explanation for this problem may be the variety of equipment used with varying parameters for beam emission area and power. These variations determine irradiation time and can directly affect experimental results [11]. While we took care to radiate the same energy density for the same area in this study, we did not observe an inhibition response that was dependent on fluence.

Table 3 Mean (standard deviation) of the power irradiation (mW) at wavelengths of 660, 830, and 904 nm over the measurement of diodes (pre- and post-experiment) and transmissibility (microtiter plate, physiologic solution bacterial strains)

	Pre-experiment	Post-experiment	Microtiter plate	Physiologic solution	*S. aureus*	*E. coli*	*P. aeruginosa*
660 nm	33.5 (0.23)	33.2 (0.22)	26.5 (0.13)[a]	32.6 (0.14)[a,b]	31.5 (0.28)[a,b,c]	31.0 (0.27)[a,b,c,d]	30.9 (0.25)[a,b,c,d]
830 nm	31.8 (0.86)	31.5 (0.18)	25.2 (0.37)[a]	31.6 (0.61)[b]	29.7 (0.34)[a,b,c]	29.8 (0.33)[a,b,c,d]	30.4 (0.23)[a,b,c,d,e]
904 nm	41.4 (0.27)	41.4 (0.86)	32.7 (0.23)[a]	41.5 (0.48)[b]	39.4 (0.44)[a,b,c]	38.2 (1.63)[a,b,c]	39.3 (0.83)[a,b,c]

$p < 0.05$ for the same wavelength

[a] Versus pre-experiment

[b] Versus microtiter plate

[c] Versus physiologic solution

[d] Versus *S. aureus*

[e] Versus *E. coli*

Among the wavelengths and bacterial species studied, red light (660 nm at 30 mW) led to the greatest bacterial inhibition, with reduced *S. aureus* growth at fluences of 12, 18, and 24 J/cm^2. In contrast, Nussbaum et al. [9] conducted a study with the same wavelength (660 nm at 75 mW) and found no significant difference in bacterial growth.

As for infrared irradiation, our results showed that *S. aureus* growth was significantly inhibited at fluences greater than 12 J/cm^2. These findings differ from our previous study [22], which showed growth inhibition at fluences of 1, 2, 3, 4, 5, and 16 J/cm^2. Furthermore, in a study by Guffey and Wilborn [13], combining wavelengths of 405 and 880 nm inhibited *S. aureus* growth in a fluence-dependent manner whereby higher fluence led to greater inhibition.

Nussbaum et al. [9] used wavelengths that were very close to those of our study and found that *S. aureus* growth was inhibited at wavelengths of 810 and 905 nm (15 mW) at a fluence of 5 J/cm^2. In contrast, a growth of 27 % was observed at a fluence of 50 J/cm^2 at a wavelength of 905 nm. In another study, the same authors evaluated the effect of laser therapy (810 nm at15 mW) at different pulse frequencies (continuous, 26, 292, 1000, and 3800 Hz) and found increased growth of *S. aureus* at the two highest frequencies [10].

When analyzing the effect of laser irradiation on *P. aeruginosa* growth, we showed that infrared light (830 nm) resulted in significant growth inhibition at fluences greater than 6 J/cm^2. However, at wavelengths of 660 and 904 nm, inhibition of *P. aeruginosa* occurred only at a fluence of 24 J/cm^2. Corroborating these findings, Guffey and Wilborn [13] obtained 93.8 % inhibition of *P. aeruginosa* growth by combining blue laser (405 nm at 200 mW) with infrared laser (880 nm at 250 mW) at a fluence of 20 J/cm^2. Similarly, Nussbaum et al. [9] obtained significant inhibition with a 810 nm laser at a fluence of 18 J/cm^2 and at wavelengths of 630 and 660 nm; *P. aeruginosa* growth decreased by 27 % (1 J/cm^2) and 18 % (5 J/cm^2), respectively. In a subsequent study, these authors observed *P. aeruginosa* growth inhibition at fluences between 5 and 50 J/cm^2 at the same wavelength and two different power settings (15 and 75 mW) [11]. However, these authors verified growth of

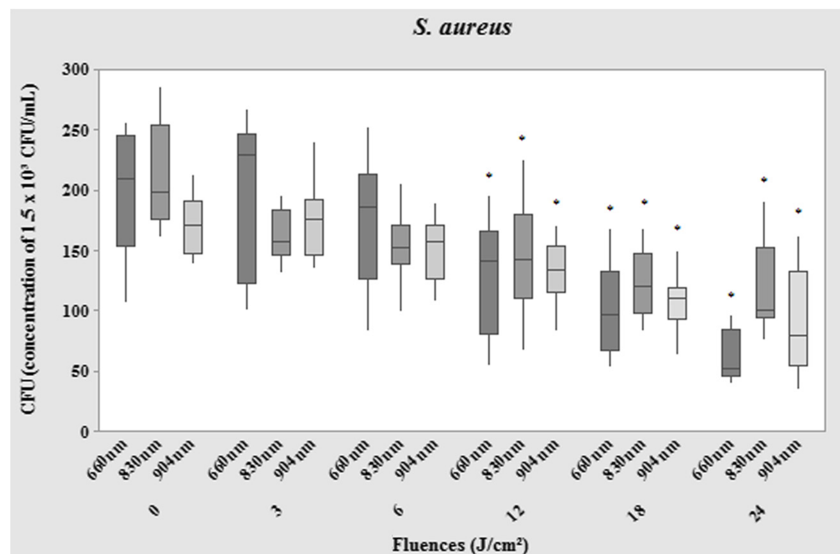

Fig. 1 *Boxplot* showing *S. aureus* growth at the times indicated after irradiation at 660, 830, and 904 nm at fluences of 0, 3, 6, 12, 18, and 24 J/cm^2

Table 4 Median (first, third quartile) of CFU (concentration of 1.5×10^3 CFU/mL) of *S. aureus*, *P. aeruginosa*, and *E. coli* irradiated at wavelengths of 660, 830, and 904 nm, at the fluences 0, 3, 6, 12, 18, and 24 J/cm^2, after 24, 48, and 48 h of the irradiation

			Control	3 J/cm^2	6 J/cm^2	12 J/cm^2	18 J/cm^2	24 J/cm^2
S. aureus	660 nm	24 h	232 (189; 256)	229 (122; 236)	148 (122; 199)	102 (77; 141)*	86 (66; 97)*	47 (45; 55)*,**,***
		48 h	228 (192; 250)	225 (120; 230)	150 (125; 196)	100 (76; 152)*	89 (67; 98)*	47 (46; 56)*,**,***
		72 h	232 (192; 248)	227 (124; 229)	151 (119; 197)	104 (76; 150)*	88 (67; 98)*	47 (46; 54)*,**,***
	830 nm	24 h	203 (194; 262)	152 (146; 159)	144 (138; 154)	140 (107; 143)*	111 (95; 122)*	97 (94; 104)*,**,#
		48 h	207 (195; 262)	155 (146; 158)	147 (135; 153)	141 (107; 146)*	109 (96; 122)*	95 (94; 101)*,**,#
		72 h	206 (197; 263)	153 (145; 158)	146 (135; 155)	140 (107; 145)*	110 (96; 122)*	96 (94; 101)*,**,#
	904 nm	24 h	191 (163; 192)	171 (146; 179)	145 (122; 157)	127 (112; 133)*	104 (84; 110)*,**	69 (52; 90)*,**,***
		48 h	186 (159; 191)	169 (146; 176)	142 (124; 160)	131 (112; 136)*	105 (91; 114)*,**	69 (54; 89)*,**,***
		72 h	183 (161; 191)	169 (145; 175)	143 (124; 161)	130 (113; 139)*	104 (91; 114)*,**	69 (54; 89)*,**,***
P. aeruginosa	660 nm	24 h	158 (144; 205)	116 (109; 126)	127 (106; 170)	114 (80; 128)	85 (60; 112)*	68 (60; 99)*,***
		48 h	148 (140; 196)	103 (98; 112)	91 (79; 143)	101 (77; 118)	83 (58; 108)*	64 (58; 99)*
		72 h	148 (139; 192)	101 (95; 111)##	88 (77; 140)##	97 (76; 115)##	82 (58; 105)*,##	63 (58; 99)*
	830 nm	24 h	169 (152; 179)	125 (108; 134)	98 (84; 111)*	106 (90; 122)*	98 (94; 109)*	89 (76; 122)*
		48 h	149 (143; 157)	114 (86; 116)	90 (78; 92)*	102 (86; 113)*	84 (82;95)*,##	80 (76; 114)*
		72 h	143 (141; 156)	112 (84; 115)##	87 (77; 90)*,##	100 (83; 113)*,##	84 (80; 92)*,##	79 (75; 114)*
	904 nm	24 h	162 (157; 183)	131 (44; 143)	106 (46; 138)	83 (47; 110)	80 (54; 121)	84 (32; 110)*
		48 h	160 (152; 179)	129 (43; 136)	102 (47; 126)	82 (46; 106)	76 (50; 108)	78 (31; 94)*
		72 h	156 (151; 176)	128 (42; 136)##	100 (47; 127)##	81 (46; 102)##,	75 (49; 105)##	77 (31; 91)*,##
E. coli	660 nm	24 h	103 (85; 125)	90 (77; 95)	94 (72; 95)	68 (60; 73)*	75 (70; 87)	94 (71; 96)
		48 h	100 (86; 120)	88 (74; 94)	91 (71; 94)	70 (58; 73)*	73 (70; 86)	94 (73; 98)
		72 h	101 (88; 122)	90 (75; 94)	90 (72; 94)	71 (59; 74)*	73 (71; 86)	94 (74; 100)
	830 nm	24 h	83 (80; 86)	59 (32; 64)*,#	52 (43; 71)*,#	47 (36; 61)*	57 (49; 72)	49 (42; 72)*,#
		48 h	84 (81; 87)	61 (30; 64)*,#	54 (42; 71)*,#	46 (35; 60)*	60 (49; 74)	47 (44; 72)*,#
		72 h	82 (80; 87)	60 (31; 64)*,#	56 (42; 71)*,#	46 (34; 60)*	60 (50; 74)	47 (44; 72)*,#
	904 nm	24 h	74 (73; 82)	80 (59; 86)	59 (51; 67)	65 (52; 76)	54 (43; 56)*,**,#	62 (55; 71)#
		48 h	74 (72; 78)	77 (58; 86)	62 (51; 68)	61 (57; 74)	53 (43; 60)*,#	61 (57; 71)#
		72 h	75 (71; 78)	78 (59; 86)	63 (50; 68)	60 (52; 75)	54 (42; 60)*,**,#	64 (58; 71)#

$p < 0.05$ in the same time and wavelength in relation to the fluence: *versus control; **versus 3 J/cm^2 ; ***versus 6 J/cm^2 . $p < 0.05$ in the same time and fluence in relation to the wavelength: #versus 660 nm. $p < 0.05$ in the same wavelength and fluence in relation to time: ##versus 24 h

P. aeruginosa at the same wavelength and fluences at different pulse frequencies (26, 1000, and 3800 Hz) [10].

Table 5 Correlation fluences/bacterial inhibition

		R_s	*P* value
660 nm	*S. aureus*	−0.8382	<0.0001
	P. aeruginosa	−0.6624	<0.0001
	E. coli	−0.2284	=0.0037
830 nm	*S. aureus*	−0.8042	<0.0001
	P. aeruginosa	−0.5223	<0.0001
	E. coli	−0.3101	=0.0003
904 nm	*S. aureus*	−0.8852	<0.0001
	P. aeruginosa	−0.4458	<0.0001
	E. coli	−0.4358	<0.0001

R_s Spearman's correlation coefficient

After irradiation, *E. coli* growth was inhibited at a wavelength of 830 nm at fluences of 3, 6, 12, and 24 J/cm^2; however, there was no difference among them. In contrast, Nussbaum et al. [10], in evaluating the effect of laser therapy (810 nm at 15 mW) in different pulse frequencies (continuous, pulsed at 26, 292, 1000, and 3800 Hz), observed growth of *E. coli* in continuous and the pulsed mode at 1000 Hz at fluences of 1, 2, 5, and 10 J/cm^2. In another study, the same authors obtained similar results using the laser in continuous mode at the same wavelength and fluences [9]. Nussbaum et al. [11] evaluated the effect of power (15 and 30 mW) on *E. coli* growth using an 810 nm laser at different fluences (1, 2, 5, 10, 20, 30, 40, and 50 J/cm^2) and observed growth at most fluences tested (1–20 J/cm^2). These results support the hypothesis that, regardless of the light spectrum, elements such as power, pulse scheme, and frequency are also determining factors for growth inhibition.

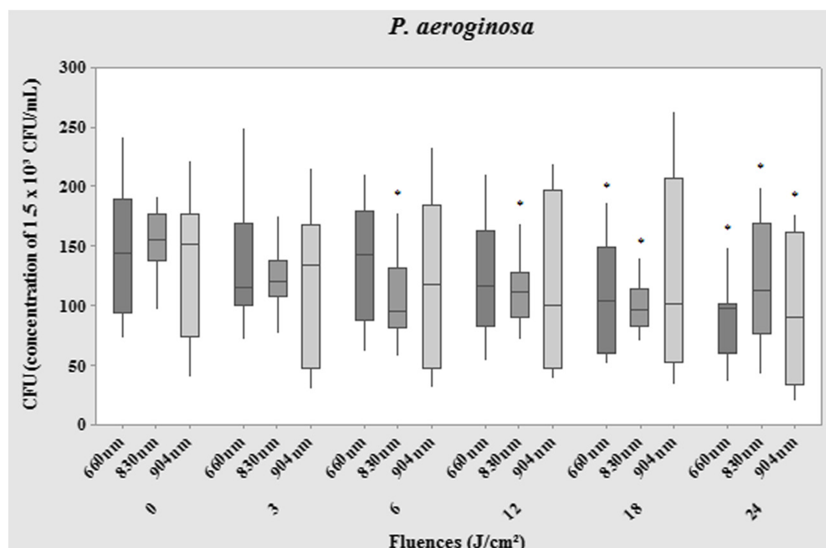

Fig. 2 *Boxplot* showing *P. aeruginosa* growth at the times indicated after irradiation at 660, 830, and 904 nm at fluences of 0, 3, 6, 12, 18, and 24 J/cm^2

In the present study, we showed that *E. coli* and *P. aeruginosa* growth were inhibited at all fluences tested, in contrast to *S. aureus* (Table 3). This can be explained by the fact that *S. aureus* are gram-positive bacteria and *E. coli* and *P. aeruginosa* are both gram-negative. Gram-positive bacteria have thick cell walls, whereas gram-negative bacteria have thinner cell walls. In addition, gram-negative bacteria have an outer membrane that contains lipopolysaccharide on the cell surface, whereas in gram-positive bacteria, the cell wall generally contains a variety of anionic substances that can bind strongly to peptides/cationic proteins, such as lipoteichoic acid (LTA) and peptidoglycans. LTA is known to be a compound of adherence in gram-positive bacteria. LTA carries the main virulence factors and initiates an inflammatory response when in contact with host cells [23]. These structural differences between gram-positive and gram-negative bacteria can impact the penetration of laser irradiation and mediate differences to irradiation susceptibility.

Another hypothesis that can be raised about the inhibitory effect of laser irradiation refers to photon absorption, by the possible chromophores present in the bacterial respiratory chain, and reactive oxygen species (ROS) can be produced, with no addition of exogenous photosensitizers preadipocytes (3T3-L1), prechondrocytes (ATDC5), myoblasts (C2C12), mesenchymal stromal cells (KUSA-A1), lung cancer cells (LLC), insulinoma cells (MIN6), fibroblasts (NIH 3T3), human cervix adenocarcinoma cells (HeLa), macrophages differentiated from lymphocytes (THP-1) after treatment with phorbol ester, and rat basophilic leukemia cells (RBL-2H3). Once, Kushibiki et al. [24] observed that intracellular chromophores have the ability to excite oxygen through the electron transport chain in eukaryotic cells. Intracellular

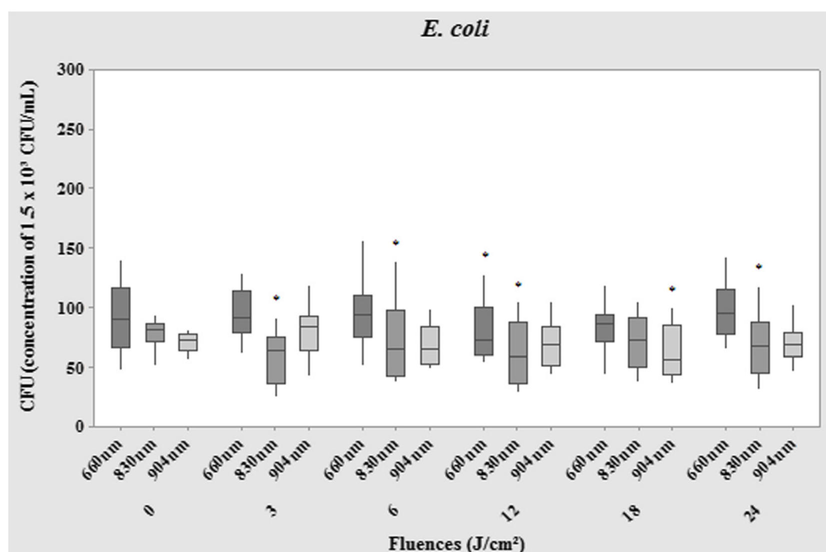

Fig. 3 *Boxplot* showing *E. coli* growth at the times indicated after irradiation at 660, 830, and 904 nm at fluences of 0, 3, 6, 12, 18, and 24 J/cm^2

porphyrin may also be responsible for ROS production [25], in particular hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$), which can promote toxic effects associated with oxidative stress, culminating in cell death [26].

Thus, these aspects should be investigated to understand morphological and physiological effects on different bacterial species, with laser irradiation at different wavelengths and fluences. Specifically, a wavelength of 450 nm should be investigated because this wavelength is known to inhibit bacterial growth [27]. These studies will be needed to reach a consensus with regard to the best parameters to be used to inhibit specific bacterial species.

Comparison of the results from this study may have been complicated by factors including the power of the diodes, irradiation time, and experimental design. In this study, the bacteria were suspended in a colorless liquid medium (saline solution) and, prior to the experiment, we showed that the saline solution led to a small decrease in irradiation transmissivity, which ensured that all content in the well of the microtiter plate was irradiated. Additionally, the temperature of the bacterial suspension remained unchanged despite a long irradiation time. However, in all studies mentioned, irradiation of bacteria was performed on culture medium of varied staining, and the laser beam absorption, refraction, and transmissivity levels were not stated [9–20]. These are important points that should be considered in future studies, since laser absorption or reflection by culture medium can affect bacterial growth and/or inhibition.

Conclusion

LLLT inhibited bacterial growth at 660, 830, and 904 nm up to 72 h after irradiation and was not time-dependent. Thus, we conclude that wavelengths of 660, 830, and 904 nm can be used to inhibit *S. aureus*, *P. aeruginosa*, and *E. coli* growth in vitro at fluences between 12 and 24 J/cm^2.

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Compliance with ethical standards

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

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