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Susceptibility of *Enterococcus faecalis* and *Propionibacterium acnes* to antimicrobial photodynamic therapy

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ABSTRACT

Bacterial resistance to available antibiotics nowadays is a global threat leading researchers around the world to study new treatment modalities for infections. Antimicrobial photodynamic therapy (aPDT) has been considered an effective and promising therapeutic alternative in this scenario. Briefly, this therapy is based on the activation of a non-toxic photosensitizing agent, known as photosensitizer (PS), by light at a specific wavelength generating cytotoxic singlet oxygen and free radicals. Virtually all studies related to aPDT involve a huge screening to identify ideal PS concentration and light dose combinations, a laborious and time-consuming process that is hardly disclosed in the literature. Herein, we describe an antimicrobial Photodynamic Therapy (aPDT) study against *Enterococcus faecalis* and *Propionibacterium acnes* employing methylene blue, chlorin-e6 or curcumin as PS. Similarities and discrepancies between the two bacterial species were pointed out in an attempt to speed up and facilitate futures studies against those clinical relevant strains. Susceptibility tests were performed by the broth microdilution method. Our results demonstrate that aPDT mediated by the three above-mentioned PS was effective in eliminating both gram-positive bacteria, although *P. acnes* showed remarkably higher susceptibility to aPDT when compared to *E. faecalis*. PS uptake assays revealed that *P. acnes* is 80 times more efficient than *E. faecalis* in internalizing all three PS molecules. Our results evidence that the cell wall structure is not a limiting feature when predicting bacterial susceptibility to aPDT treatment.

1. Introduction

Bacterial resistance to available antibiotics and the low perspective related to the discovery of new drugs have lead researchers to search for alternative antimicrobial treatment [1], and photodynamic therapy (PDT) is among the alternatives. PDT is a treatment modality based on the interaction of three fundamental components: a photosensitizer (PS), visible light and oxygen [2,3]. When the PS is excited by the light it can interact with the surroundings through two pathways, named type I and type II reactions. Type I reaction takes place when the PS in its excited triplet state transfers charges (e^- or H^+) to biomolecules, to originate radical species. In the type II reaction, the triplet excited state PS transfers energy directly to the ground-state triplet oxygen, in a phenomenon named triplet-triplet annihilation, leading to the formation of the highly reactive and cytotoxic singlet oxygen. Both reactions take place at the same time and the relation between the two processes depends on the PS, the oxygen and substrates concentrations, and the bond-affinity of PS with the substrates [4–7].

Photosensitizers are molecules that possess the ability to absorb light in specific wavelengths and use the energy to trigger photo-oxidative reactions in the presence of molecular oxygen [2,8]. An efficient PS must present no toxicity in the absence of light activation, selective uptake by the target cell, and generate high amounts of reactive oxygen species (ROS), among other properties [9]. In the past few years, different PSs have been studied in PDT. Curcumin (CUR), methylene blue (MB), and chlorin-e6 (Ce6) are examples of molecules with well-established photosensitizing properties, which are being widely employed in studies involving this treatment modality [10–14], and were the PS

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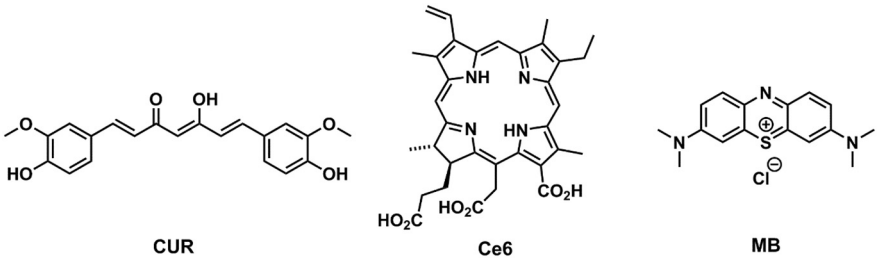

Fig. 1. Chemical structures of the photosensitizers. CUR: curcumin; Ce6: chlorin-e6; MB: methylene blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used in this study.

The three PS were chosen for their distinct chemical properties (Fig. 1), allowing us to evaluate if the aPDT effects would present some level of selectivity regarding the PS molecule. Curcumin, for example, is a phenolic natural product that can be synthetically obtained or extracted from the rhizomes of *Curcuma longa*. This natural PS is a medium-water insoluble molecule and presents an absorption band mostly in the blue region, with a peak at 430 nm [15–17]. Interestingly, the fast photobleaching of CUR allows the treatment of local lesions and infections with no persistent photosensitizing activity after a few hours, making this PS very strategic.

Methylene blue, by its turn, is a low-cost phenothiazine derivative, well-soluble in water and ethanol, with a maximum absorption at 664 nm, a wavelength with good tissue penetration, which enables the application of MB to treat deeper lesions [18,19]. Finally, chlorin-e6 belongs to the class of chlorophyll derivatives, with its tetrapyrrole ring conferring it hydrophobic and hydrophilic (amphiphilic) features; it absorbs light in both 400 and 660 nm and produces high amounts of singlet oxygen, both *in vitro* and *in vivo* [20–22]. Structurally related to PDZ® (Photoditazine), Ce6 has been presenting better PDT activities due to its amphiphilicity and absence of dark toxicity [23] and is considered as a very promising candidate for systemic and non-systemic treatments. In summary, our study covered different classes of PS with different chemical and photochemical properties.

Numerous studies suggest that Gram-positive bacteria are more sensitive to aPDT than Gram-negative, due to the differences in their cell wall structure. Gram-positives present a thick peptidoglycan layer over the plasmatic membrane, which confers them a relatively more porous cell wall, allowing for the PS to diffuse easily to the cell interior. Gram-negatives, on the other hand, have a highly selective external membrane, a thin peptidoglycan layer, and the plasmatic membrane, making the PS permeation more difficult [24–27].

However, studies have demonstrated that the parameters used to determine the bacterial susceptibility to aPDT, such as light dose and PS concentration, can vary independently of the cell wall structure, with discrepant differences of parameters and response to the therapy found among bacteria of the same coloration group, evidencing that the cellular structure is not the limiting factor for the success of the therapy [28–30]. *Propionibacterium acnes* and *Enterococcus faecalis*, for instance, are both Gram-positive non-spore forming facultative anaerobes [31] whose susceptibility to aPDT have been demonstrated to be remarkably different, considering the same PS, in studies of our group. This finding suggests a more complex mechanism of susceptibility than just the cell wall structure, given that both species present the same basic assembly.

After several studies with both species conducted by our group, we observed a huge necessity to adapt the initial aPDT screening for PS concentration and light dose combinations for each one, even though they are both gram-positive. In general, data obtained from aPDT screenings are not disclosed in publications, having the sole purpose of providing ground information for other assays. This pattern of disclosing, however, implies that every new study has to initiate a new screening until determining the ideal combinations of PS concentration and light dose. Therefore, one of the aims of the present study was to provide basic parameters to facilitate future studies with those strains, as well as to investigate the mechanisms behind the difference in behavior observed for the two species.

2. Material and Methods

2.1. Bacterial Strains and Culture Conditions

The strains used in this study were *Enterococcus faecalis* (ATCC® 29,212™) and *Propionibacterium acnes* (ATCC® 6919™), both obtained from the National Institute of Quality Control in Health (INCQS) from the Oswaldo Cruz Foundation (FIOCRUZ - Manguinhos, RJ, Brazil). *E. faecalis* was cultured in blood agar (defibrinated sheep blood 5%; BHI 2,6%; TSA 2%; yeast extract 1%) in an anaerobic jar at 37 °C; *P. acnes* was incubated under the same conditions, but in Reinforced Clostridial Agar (Himedia - Mumbai, India). Strains were cultured in solid media and isolated colonies were suspended in TSB (Kasvi - Curitiba, PR, Brazil; *P. acnes*) or BHI broth (Kasvi - Curitiba, PR, Brazil; *E. faecalis*) to prepare the inocula prior to each assay. Inocula were adjusted by spectrophotometer (Biotek® ELx800 - Winooski, VT, USA) reading at 630 nm to yield an initial suspension of $\sim 5 \times 10^9$ cell/mL.

2.2. Photosensitizers and Light Sources

Chlorin-e6 (Ce6) and curcumin (CUR) were synthesized as described in the literature [32–34]. Methylene blue (MB) was obtained from Sigma (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). All three photosensitizers' solutions were prepared in an environment protected from light. A stock solution of methylene blue was prepared in deionized water and diluted to working concentrations in broth; curcumin's stock solution was prepared in 100% DMSO and diluted in 0.5% sucrose to achieve working concentrations; chlorin-e6 was solubilized in 10% DMSO to give stock solutions, and diluted to working concentrations in broth. Work solutions were always prepared immediately prior to the beginning of the assays.

Light sources consisted of 48 LEDs with variable intensities assembled as a compact illumination system with a homogeneous illumination area and a cooling system to avoid overheating (IrradLED® – biopdi, Sao Carlos, SP, Brazil). Curcumin was activated by light at 450 nm (151 mW/cm^2); methylene blue and chlorin-e6 were activated by light at 660 nm (153 mW/cm^2). The distance between the LEDs and the plate allowed an even distribution of light on each well, and light was delivered from underneath the plates.

2.3. Photodynamic Therapy

Susceptibility test was performed by the broth microdilution technique, employing a 96 wells plate. PS solutions were prepared with twice the desired work concentration, in triplicates, to a final volume of 50 μL/well. Bacterial suspensions were prepared in 3 mL of broth (BHI for *E. faecalis*, TSB for *P. acnes*), their optical density was measured at 630 nm (OD_{630} between 0.08 and 0.1) and 50 μL aliquots were added to each well already containing a PS solution or 50 μL of broth (growth control). The addition of the suspensions to the wells resulted in a 50% dilution of both the inocula and the solutions. Bacterial suspensions

Table 1
– chlorin-e6-mediated aPDT.

Strain	Chlorin-E6 concentration	Light dose	Average log_{10} of CFU/mL (SD)	Log_{10} reduction[a]	% reduction[a]
Enterococcus faecalis	21 μM	30 J/cm^2	9.56 (0.56)	0.64	61.67
		45 J/cm^2	9.26 (0.08)	0.93	86.79
		60 J/cm^2	8.84 (0.08)	1.36	95.00
		90 J/cm^2	8.66 (1.14)	1.54	97.00
	42 μM	30 J/cm^2	9.70 (0.46)	0.50	51.90
		45 J/cm^2	8.39 (0.07)	1.81	98.21
		60 J/cm^2	0.00 (0.00)	10.20	100
		90 J/cm^2	0.00 (0.00)	10.20	100
	84 μM	30 J/cm^2	9.47 (0.86)	0.73	55.00
		45 J/cm^2	0.00 (0.00)	10.20	100
		60 J/cm^2	0.00 (0.00)	10.20	100
		90 J/cm^2	0.00 (0.00)	10.20	100
	126 μM	30 J/cm^2	9.73 (0.24)	0.47	58.57
		45 J/cm^2	0.00 (0.00)	10.20	100
		60 J/cm^2	0.00 (0.00)	10.20	100
		90 J/cm^2	0.00 (0.00)	10.20	100
	168 μM	30 J/cm^2	9.51 (0.66)	0.69	61.79
		45 J/cm^2	0.00 (0.00)	10.20	100
		60 J/cm^2	0.00 (0.00)	10.20	100
		90 J/cm^2	0.00 (0.00)	10.20	100
Propionibacterium acnes	2.62 μM	3.25 J/cm^2	0.00 (0.00)	8.20	100
		7.5 J/cm^2	0.00 (0.00)	8.20	100
		15 J/cm^2	0.00 (0.00)	8.20	100
	5.25 μM	3.25 J/cm^2	0.00 (0.00)	8.20	100
		7.5 J/cm^2	0.00 (0.00)	8.20	100
		15 J/cm^2	0.00 (0.00)	8.20	100
	10.5 μM	3.25 J/cm^2	0.00 (0.00)	8.20	100
		7.5 J/cm^2	0.00 (0.00)	8.20	100
		15 J/cm^2	0.00 (0.00)	8.20	100
	21 μM	3.25 J/cm^2	0.00 (0.00)	8.20	100
		7.5 J/cm^2	0.00 (0.00)	8.20	100
		15 J/cm^2	0.00 (0.00)	8.20	100
	42 μM	3.25 J/cm^2	0.00 (0.00)	8.20	100
		7.5 J/cm^2	0.00 (0.00)	8.20	100
		15 J/cm^2	0.00 (0.00)	8.20	100

[a] Compared to control.

were incubated with the PS for 5 (curcumin and methylene blue) or 10 min (chlorin-e6) prior to plate irradiation with the appropriate light dose. After treatment, suspensions were submitted to 10-fold serial dilutions until reaching ~10^3 cell/mL (*P. acnes*) or ~10^1 cell/mL (*E. faecalis*), and 5 μL of each suspension was plated. The number of grown colonies was accessed after 2 days of growth at 37 °C in an anaerobic jar.

2.4. Photosensitizer Uptake

Bacterial suspensions (~10^8 cells/mL) were incubated in BHI or TSB broth at room temperature in the dark for 5 min with the photosensitizer in the following concentrations: Ce6–21 and 42 μM; CUR – 17 and 34 μM; MB – 39 and 78 μM. Controls comprised of suspensions incubated with broth only. All groups were assayed in triplicates, on three different occasions. After incubation, cell suspensions were centrifuged (6500 rpm for 5 min), the supernatant was discarded, and bacteria were washed twice with 1 mL of sterile phosphate buffer (0.1 M, pH 7.4), and centrifuged again. The resulting bacterial pellet was dissolved in 2 mL of 0.1 M NaOH-1% sodium dodecyl sulfate (SDS) for 24 hate room temperature for cell digestion. The extracts had their endpoint fluorescence read in a spectrofluorometer (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA). For MB, the excitation wavelength was 660 nm and the emission was 690 nm; for Ce6, excitation was 500 nm and emission was 670 nm; for CUR, excitation was 450 nm and emission was 640 nm. PS dissolved in NaOH-SDS in several concentrations were used to make calibration curves, which were used for determination of PS concentration in the extract. PS concentration uptaken was correct by the cell number.

2.5. Statistical Analysis

Data were expressed as the mean plus standard deviation (SD) and were analyzed by one-way ANOVA with Tukey's *post hoc* test, using GraphPad Prism® Version 5.01 software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be significant when $p < 0.05$ (confidence level of 95%) and the maximum acceptable coefficient of variation was 25%.

3. Results

3.1. aPDT Susceptibility

A screening comprising several PS concentrations *versus* energy fluences was conducted for both strains. Tables 1, 2 and 3 summarize the results. By analyzing the results of aPDT with chlorin-e6 (Ce6; Table 1) it is evident that *P. acnes* is extensively more susceptible to the treatment than *E. faecalis*. The minimum light dose applied to *E. faecalis* was 30 J/cm^2, which resulted in a maximum bacterium reduction of 61.8% (< 1 log_{10} of CFU/mL) when employing 168 μM of Ce6. By applying a roughly 10-fold lower energy fluence to *P. acnes* (3.25 J/cm^2), however, complete bacterial elimination was achieved with 2.62 μM of Ce6. In comparison, the minimum Ce6 concentration to result in total *E. faecalis* reduction was 42 μM irradiated with 60 J/cm^2.

The results obtained from aPDT with curcumin (Table 2) were very similar to the ones described for Ce6, with *P. acnes* presenting higher susceptibility to the therapy compared to *E. faecalis*. By employing 12.5 J/cm^2, only 14 μM of curcumin was necessary to completely eliminate *P. acnes,* whereas the same result for *E. faecalis* required 136 μM of the photosensitizer. In fact, as for Ce6, all curcumin *versus*

Table 2
– curcumin-mediated aPDT.

Strain	Curcumin concentration	Light dose	Average log_{10} of CFU/mL (SD)	Log_{10} reduction[a]	% reduction[a]
Enterococcus faecalis	17 μM	12.5 J/cm^2	10.12 (0.27)	0.20	33.24
		18.75 J/cm^2	10.14 (0.19)	0.18	36.69
		25 J/cm^2	10.15 (0.14)	0.16	36.03
	34 μM	12.5 J/cm^2	10.10 (0.41)	0.22	36.56
		18.75 J/cm^2	10.02 (0.29)	0.29	48.38
		25 J/cm^2	9.74 (0.51)	0.57	68.53
	68 μM	12.5 J/cm^2	10.08 (0.03)	0.23	46.40
		18.75 J/cm^2	9.23 (0.66)	1.08	84.89
		25 J/cm^2	0.00 (0.00)	10.32	100
	136 μM	12.5 J/cm^2	0.00 (0.00)	10.32	100
		18.75 J/cm^2	0.00 (0.00)	10.32	100
		25 J/cm^2	0.00 (0.00)	10.32	100
Propionibacterium acnes	14 μM	3.12 J/cm^2	0.00 (0.00)	8.45	100
		6.25 J/cm^2	0.00 (0.00)	8.45	100
		12.5 J/cm^2	0.00 (0.00)	8.45	100
	27 μM	3.12 J/cm^2	0.00 (0.00)	8.45	100
		6.25 J/cm^2	0.00 (0.00)	8.45	100
		12.5 J/cm^2	0.00 (0.00)	8.45	100
	54 μM	3.12 J/cm^2	0.00 (0.00)	8.45	100
		6.25 J/cm^2	0.00 (0.00)	8.45	100
		12.5 J/cm^2	0.00 (0.00)	8.45	100

[a] Compared to control.

Table 3
– methylene blue-mediated aPDT.

Strain	Methylene blue concentration	Light dose	Average log_{10} of cfu/ml (sd)	Log_{10} reduction[a]	% reduction[a]
Enterococcus faecalis	39 μM	30 J/cm^2	9.84 (0.03)	0.15	29.05
		60 J/cm^2	9.96 (0.17)	0.03	27.97
		90 J/cm^2	9.96 (0.67)	0.02	57.91
	78 μM	30 J/cm^2	9.98 (0.15)	0.01	23.96
		60 J/cm^2	9.69 (0.09)	0.30	49.44
		90 J/cm^2	9.35 (0.04)	0.63	76.58
	156 μM	30 J/cm^2	9.88 (0.10)	0.11	21.49
		60 J/cm^2	9.40 (0.31)	0.59	70.84
		90 J/cm^2	8.93 (0.21)	1.05	90.53
	312 μM	30 J/cm^2	9.68 (0.02)	0.31	51.09
		60 J/cm^2	8.31 (0.00)	1.69	97.96
		90 J/cm^2	0.00 (0.00)	9.98	100
Propionibacterium acnes	39 μM	30 J/cm^2	8.12 (0.17)	0.00	0.00
		60 J/cm^2	8.09 (0.24)	0.11	26.65
		90 J/cm^2	7.84 (0.14)	0.36	55.57
	78 μM	30 J/cm^2	8.12 (0.16)	0.08	24.72
		60 J/cm^2	8.07 (0.15)	0.04	25.82
		90 J/cm^2	7.65 (0.17)	0.55	70.78
	156 μM	30 J/cm^2	8.09 (0.18)	0.11	28.57
		60 J/cm^2	7.14 (0.33)	0.97	89.59
		90 J/cm^2	0.00 (0.00)	8.20	100

[a] Compared to control.

light dose combinations resulted in complete *P. acnes* elimination.

Table 3 shows the results obtained from aPDT employing methylene blue as the PS. *P. acnes* was less susceptible to aPDT with MB compared with the other photosensitizers, but remained more sensitive than *E. faecalis*, which is evidenced by the concentration of MB required to completely eliminate both strains, using the same light dose: *P. acnes* colony forming units (CFU) were eliminated with half the concentration (156 μM) necessary to eliminate *E. faecalis* CFU (312 μM).

3.2. Photosensitizer Uptake

The difference in susceptibility observed between the two strains could be a result of differential uptake of the photosensitizers; therefore, the ability to uptake each PS was investigated for both strains, using two concentrations of each photosensitizer. As can be seen in Fig. 2, *P. acnes* (averages around 8×10^{-9}) uptakes 80 times more PS than *E. faecalis* (averages around 1×10^{-10}), evidencing that the higher susceptibility of *P. acnes* to aPDT is a result of superior accumulation of PS molecules inside the cell, which directly affects the concentration of ROS generated in lethal locations within the bacterium. Moreover, increasing the initial concentration of the PS administered to the bacteria does not affect the concentration of PS internalized, revealing that (i) there is a saturation of the system and (ii) that the light dose plays a major role in the success of PDT.

4. Discussion

Bacteria composing the *Enterococcus* genera are related to the majority of hospital infections, being the third most isolated nosocomial pathogen. Although several species might be related to diseases, most enterococcal infections in humans are caused by *E. faecalis* [35,36], including endodontic, urinary tract, and wound infections, endocarditis, and bacteremia [37]. Infections caused by vancomycin-resistant *E. faecalis* are of particular concern because the genes conferring

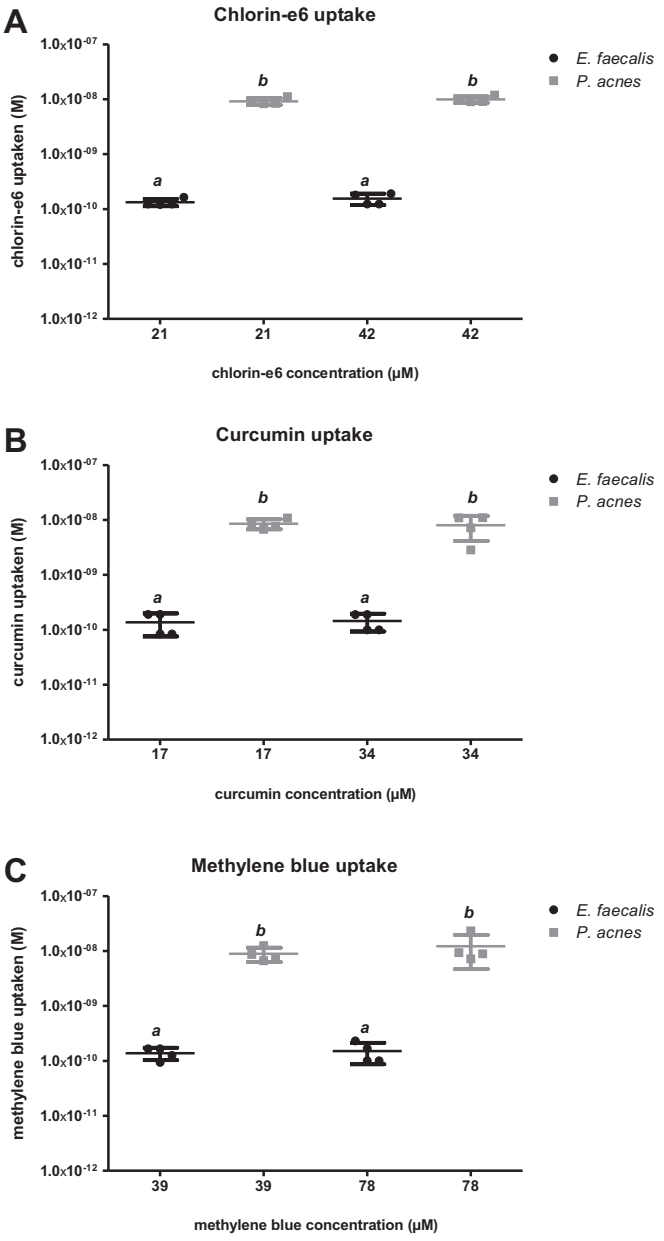

Fig. 2. Photosensitizer uptake assays. Standardized suspensions of *Enterococcus faecalis* or *Propionibacterium acnes* were incubated with chlorin-e6 (21 or 42 μM; A), curcumin (17 or 34 μM; B) methylene blue (39 or 78 μM; C), for 5 min in the dark. The lines inside represent the averages with standard deviation. Four independent assays (n = 12). Different letters note statistical difference among groups. One-way ANOVA with Tukey *post-hoc*. MB: methylene blue; Ce6: chlorin-e6; CUR: curcumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

it resistance are located in mobile genetic elements, such as plasmids and transposons, making the spread of resistance to other strains especially easy [38].

P. acnes, by its turn, is an opportunistic pathogen highlighted in the literature by being considered the main causative microorganism of acne vulgaris [39], a chronic skin disease that can result in disfiguring scars, usually accompanied by severe psychological impact on patients [40]. To a minor extent, *P. acnes* is also related to other infections, including osteomyelitis, endophthalmitis, endocarditis, joint prostheses infections, and endodontic infections [41–44]. In recent years, reports revealing increasing antimicrobial resistance of *P. acnes* to antimicrobials used for the treatment of acne vulgaris have grown in number [45].

The clinical relevance of both strains, together with their increasing resistance o antimicrobials, grounds the importance of pursuing new therapeutic strategies to manage infections caused by them. Within the limitations of this *in vitro* study, aPDT mediated by the three PS was effective in eliminating *E. faecalis* and *P. acnes*. Although both bacteria present the same basic cell wall architectures, we observed that the parameters necessary to reduce the microbial load were very different between the two species. *P. acnes* was lethally susceptible to all combinations of light dose and Ce6 or CUR concentrations. To completely eliminate *E. faecalis*, however, it was necessary to apply a Ce6 concentration 16-fold higher and a light dose 18-fold higher; for CUR-PDT, *E. faecalis* required almost 5 times more CUR and 8 times more light energy. Those results evidenced that *P. acnes* is particularly more susceptible to CUR-PDT and Ce6-PDT than *E. faecalis*. Regarding MB-PDT, *E. faecalis* required only twice the concentration of MB than *P. acnes*, demonstrating that both strains have similar susceptibility to this cationic PS.

As our results revealed, *P. acnes* internalizes almost 100 times more PS than *E. faecalis*, regardless the molecule or its administered concentration. Since ROS have a short radius of action (~2 nm in average), the site of generation of those species once the PS is activated by the light is a key factor for the success of PDT, which means that the specific localization of the molecule inside the cell (*i.e.*, associated with DNA, proteins or the cytoplasmic membrane) directly influences the efficiency of a photosensitizer and is strictly related to its chemical nature [46,47]. Therefore, even though all three PS were internalized at similar rates within one strain, they presented different efficiency (Ce6 > CUR > MB) most likely due to their distinct affinity to intracellular molecules.

5. Conclusion

According to the methodology employed in this *in vitro* study, aPDT mediated by curcumin, chlorin-e6 or methylene blue is an effective alternative to eliminate both *Enterococcus faecalis* and *Propionibacterium acnes*. *P. acnes* showed remarkably higher susceptibility to aPDT when compared to *E. faecalis,* which was revealed to be a result of differences in PS internalization by the two strains. Our results evidence that susceptibility to aPDT cannot be predicted solely based on the cell wall structures of bacteria.

Author's Disclosure of Potential Conflicts of Interest

The authors declare that there are no potential conflicts of interest.

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