Universidade de Aveiro Departamento de Biologia
2016

Tatiana Moura Branco

Combining Antibiotics and Photodynamic Therapy to inactivate *Staphylococcus aureus* on skin

Combinação entre Antibióticos e Terapia Fotodinâmica para inativar *Staphylococcus aureus* na pele

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Maria do Amparo Ferreira Faustino, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Palavras-chave

Porfirinas, Terapia Fotodinâmica Antimicrobiana, *Staphylococcus aureus*, Antibióticos, Pele.

Resumo

Staphylococcus aureus é uma bactéria de Gram-positivo que está comumente presente em infeções da pele podendo espalhar-se através da corrente sanguínea e afetar outros órgãos. Para o tratamento destas infeções são normalmente utilizados os antibióticos, no entanto, os microrganismos têm a capacidade de adquirir resistência aos agentes antimicrobianos. A terapia fotodinâmica antimicrobiana está a ser estudada ativamente como alternativa ao tratamento de infeções localizadas. Este estudo foi orientado para avaliar a atividade antibacteriana da terapia fotodinâmica no tratamento de infeções por *S. aureus* na superfície da pele. Foi também avaliado o efeito sinérgico da terapia fotodinâmica antimicrobiana e de antibióticos (ampicilina, cloranfenicol, canamicina, penicilina G e tetraciclina) na inativação de *S. aureus*. Para este fim, foi utilizado uma porfirina tetra catiónica (Tetra-Py^{+}-Me) que foi testada *in vitro* para inativar a bactéria numa solução tampão e *ex vivo* em pele de porco artificialmente contaminada com *S. aureus*. Os resultados mostraram inactivação eficaz de *S. aureus* (redução de 8 log) em tampão fosfato salino utilizando a porfirina Tetra-Py^{+-}Me numa concentração de 5.0 µM após 180 minutos de irradiação com luz branca (com incidência de 40 $W.m^{-2}$) Na combinação de ampicilina (MIC 0,25 µg mL^{-1}) em concentrações de 0,5 µg mL^{-1} e 1 µg mL^{-1} com a porfirina Tetra-Py^{+-}Me a 5.0 µM (em tampão fosfato salino) foi observado um decréscimo mais rápido (8 log) na inativação total do número de bactérias após 30 e 60 min de irradiação, respetivamente. Para os outros antibióticos não foi observado qualquer aumento na inactivação bacteriana. Nos ensaios *ex vivo* houve uma redução de ~4 log após tratamento com a porfirina Tetra-Py^{+}-Me a 50 µM após 180 minutos de irradiação. A eficiência da inativação na pele nas mesmas condições, mas na presença de 5 µg mL^{-1} de ampicilina foi significativamente diferente da obtida com PS na ausência do antibiótico com uma inativação de ~5,6 log. Os resultados deste estudo mostraram que a DPT é uma abordagem eficaz para controlar infeções por *S. aureus* na pele, inativando as bactérias até ao limite de deteção após três ciclos de tratamento. Além disso, a combinação de aPDT com antibióticos pode aumentar a eficácia da inactivação bacteriana, permitindo a redução do tempo de tratamento para um quarto.

Keywords Porphyrins, Antimicrobial Photodynamic Therapy, *Staphylococcus aureus*, Antibiotics, Skin.

Abstract

Staphylococcus aureus is a Gram-positive bacterium common in skin infections, but this bacterium can spread through the bloodstream and infect distant organs. To the treatment of this infections antibiotics are usually used, however, microorganisms have acquired the capacity to develop resistance against antimicrobial agents. Antimicrobial photodynamic therapy (aPDT) is being actively studied as a possible alternative to antibiotics to treat localized infections. This study was conducted to evaluate the antibacterial activity of aPDT for treatment of *S. aureus* infections on skin. The synergistic effect of aPDT and antibiotics (ampicillin, chloramphenicol, kanamycin, penicillin G and tetracycline) to inactivate *S. aureus* was also evaluated. To this purpose, a tetracationic porphyrin, the 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) was used to inactivate *S. aureus in vitro* using a buffer solution (PBS) and *ex vivo*, on pork skin artificially contamined with *S. aureus*. The results show an efficient inactivation of *S. aureus* in PBS using 5.0 μM of Tetra-Py^+-Me during 180 min in the presence of a white light at an irradiance of 40 W m^{-2} (reduction of 8 log). When aPDT was done in the presence of ampicillin at 0.5 and 1.0 μg mL^{-1} (MIC dose 0.25 μg mL) in PBS a faster decrease (8 log) in total bacterial number was observed at 60 and 30 min, respectively. For the other antibiotics no increase in bacterial inactivation was observed. In *ex vivo* experiments a reduction of ~4 log of *S. aureus* after treatment with 50 μM of Tetra-Py^+-Me under after 180 min. The efficiency of inactivation in the skin in the same conditions but in the presence of 5 μg mL^{-1} of ampicillin at 50 μM was significantly different of that obtained with PS in the absence of antibiotic with an inactivation of ~5.6 log. The results of this study showed that aPDT is an effective approache to control *S. aureus* infection in skin, inactivating the bacteria to the detection limit after three cycles of treatment. Moreover, the combination of aPDT with antibiotics can increase the efficacy of bacterial inactivation, allowing the reduction the treatment time for a quarter.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

$^1O_2$	Singlet oxygen
$^3O_2$	Molecular oxygen in the ground state
AMP	Ampicillin
ANOVA	Analysis of variance
aPDT	Antimicrobial Photodynamic Therapy
BHI	Brain-Heart Infusion
BPA	BD Baird-Parker Agar
BPA	BD Baird-Parker Agar
CFU	Colony-forming Unit
CLOR	Chloramphenicol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
Gram (–)	Gram-negative
Gram (+)	Gram-positive
HpD	Hematoporphyrin derivative
J	Joule
KAN	Kanamycin
log	Logarithm
MIC	Minimum Inhibitory Concentration
O_2	Molecular oxygen
ºC	Degree Celsius
PBS	Phosphate-buffered Saline
PDI	Photoinactivation
PDT	Photodynamic Therapy
PEN G	Penicillin G
PRT	Photoradiation Therapy
PS	Photosensitizer
rpm	Revolutions per minute
TETRAC	Tetracycline
Tetra-Py^+-Me	5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide

TSA	Trypticase Soy Agar
W	Watt

THESIS OUTLINE

The objective of this study was to evaluate the antibacterial activity of aPDT for the treatment of *Staphylococcus aureus* infections on skin and also to assess if aPDT efficacy increase in the presence of antibiotics.

For this, the photodynamic effect on *S. aureus* using a tetracationic porphyrin as photosensitizer (PS) Tetra-Py^{+}-Me at different concentration (5.0 µM, 25 µM, 40 µM, 50 µM) and two artificial white light of irradiances of 40 W m^{-2} and 150 W m^{-2}) was evaluated. The photodynamic inactivation was evaluated *in vitro* and *ex vivo*. The assay in *ex vivo* was produced on skin of porcine, which has served as a model for human tissuet. The efficiency of photodynamic therapy combinated with different antibiotics (ampicillin, chloramphenicol, kanamycin, penicillin G and tetracycline) was also tested in the same conditions.

Chapter 1 consists on a brief background, focusing on the photodynamic therapy, the antimicrobial photodynamic therapy, the mechanisms of photodynamic inactivation, types of antibiotics used for treatment of infections by *S. aureus* and some mechanisms of resistance of antibiotics.

Chapter 2 describes the experimental work, the results obtained and their discussion as well as the main conclusions.

CHAPTER 1

1. Photodynamic Therapy

1.1. Briefly History

The benefits, for restoration of health, of exposure to sunlight was known in centuries [1,2] and it association with photosensitizers has been used for centuries from the ancient civilizations of Egypt, Greece and India. In fact, they used the photodynamic action by the ingestion of plants containing for instance psoralens which in combination with the exposure to sunlight treated skin disorders such as vitiligo, psoriasis and rickets [3,4].

With the understanding of the photodynamic mechanism, von Tappeiner and colleagues performed the first PDT trial in patients with skin carcinoma using eosin as photosensitizer (PS), applied topically together with the application of sunlight [3,5].

The success of this and other tests conducted, in 1993 in Canada, the semi-purified preparation of HpD known as the Photofrin® (porfimer sodium), to be the first regulatory approval PS for the treatment of bladder cancer. This was later, in 1995, approved by the Food and Drug Administration (FDA) for the treatment of esophageal cancer, and later to be used in several countries in Europe. Today, there are already several PS approved in several countries for applications in PDT [1,5,6]. The PDT, appears in the past years as a viable alternative, cost efficient and with promising applications in several areas, namely, as an antimicrobial approach, in clinical field, food industry and environmental control [7,8].

1.2. Principle and mechanisms of Photodynamic Therapy

Photodynamic therapy is a therapeutic modality requiring the combined action of three main components in promoting cytotoxicity: a PS, a visible light source (a coherent laser light or a non-coherent one, for instance, an LED or Xenon lamp), and the molecular oxygen usually present in the biological target at an affordable concentration [9]. PDT is based on the visible light activation of a PS molecule in the presence of molecular oxygen which results in generation of reactive oxygen species (ROS) [10–12].

Reactive oxygen species (ROS) is a collective term used for oxygen derived free radicals (superoxide, hydroxyl radical, nitric oxide) and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite) [13,14]. The mechanisms of photosensitization involve generation of singlet oxygen ($^1O_2$) via energy transfer from the triplet excited state of PS to the molecular oxygen which is always abundant in a biological environment (Type II) or via electron or hydrogen transfer from the triplet excited state of PS to surrounding molecules (Type I) [15–18]. The photophysical process is illustrated in Figure 1, using the energy levels or Jablonski diagram, illustrated the states according to their energy as well as spin multiplicity [16].

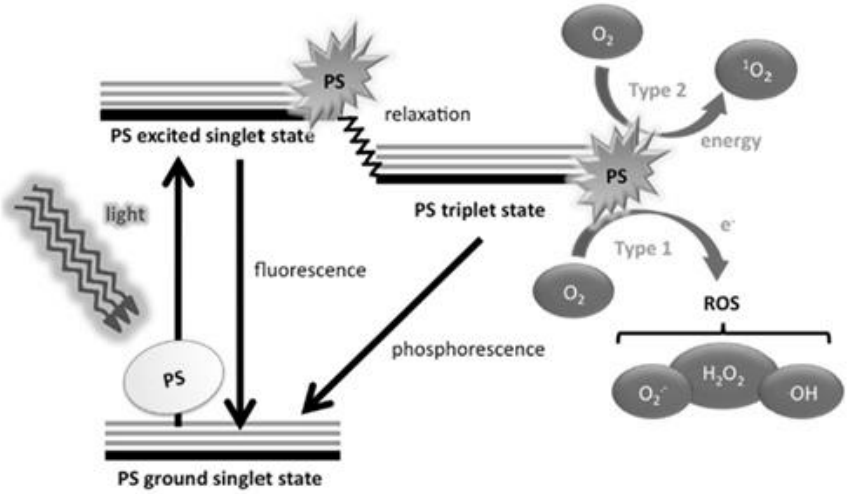


Figure 1 – Scheme of photosensitization [14].

One essential property of a good PS is a high intersystem crossing (ISC) yield, a high probability of transition from S_1 to an excited triplet state T_1 [17]. The excited triplet state is the main mediator of the photodynamic reactions. In the T1 state, the photosensitizer can transfer energy to molecular oxygen ($^3O_2$), exciting it to its highly reactive singlet state ($^1O_2$). During the energy transfer process the PS is simultaneously brought back to its singlet ground state (S_0) where it can, in principle, take part in further sensitisation cycles [16,17].

The basic protocol of PDT involves PS administration followed by a wait time of varying duration to allow for the accumulation of the PS in the cells/tissue, after which the target tissue is irradiated with light [11,19]. The irradiation activates the PS in singlet ground state (S_0) to transit from a low power state into a singlet excited state (S_1). In this case, there

are a transit of electrons to a different orbital, exciting the PS to the form of an unstable molecule with a short half-life (first excited singlet-state, S_1) [17,20]. In order to return to its stable ground state, the PS emits fluorescence or phosphorescence [21]. Fluorescence emission does not alter the electron spin but phosphorescence changes [10]. In other hand, the unstable molecule can transit to triplet state (T_1) and transfers energy with the production of ROS as it was mentioned before.

In aPDT, the high levels of ROS, surrounding pathogenic microorganisms lead to cellular damage, specifically oxidate lipids, peptides or nucleic acids [10,22]. Those can elicit either cell survival or dead depending on severity and duration of exposure [10,23,24]. These radicals reduces the probability of the selection of resistant strains, which is the main problem faced by the current conventional antibacterial therapies [25–29] .

1.3. Light Source

The efficiency of PDT requires an adequate light that activates the used PS. This light must be monochrome and centered on PS absorption band used [30]. The first light sources to be used were conventional bulbs with no coherent and polychromatic light [31]. Since the emergence of the first laser equipment and LEDs, those light sources have been more used in PDT due to its properties: high concentration of energy, low energy dispersion, coherence and monochromaticity, possibility of lighting a medium composed of different materials and only interaction with a particular component (selectivity) [32]. The wavelength of light used for PDT is typically in the wavelength range between 600–800 nm, the so called “therapeutic window” which corresponds to a depth of light penetration 0.5 to 1.5 cm [17].

The dosimetry of light is a major factor in the effectiveness of treatment and depends on the area and depth of the lesion [33]. This parameter is usually expressed by the light irradiance, which is defined in terms of energy incident on a given area of lesion per unit time ($W.m^{-2}$, $W=J.s^{-1}$) [34].

The clinical efficacy of PDT dosimetry will also depends on illuminated system used: the total dose and irradiation time delivery mode (continuous or fractionated irradiation) [35]. The use of optical fibers allowed the light to be directed easily to deliver irradiation to desired regions without the requirement of a straight light path [17].

1.4. Photosensitizers (PS)

PS are usually aromatic molecules that can be a natural or a synthetic nature which may absorb light of certain energy and may undergo an electronic transition to the singlet excited state (electron spins paired) after interaction with an appropriate light [35]. Different PSs have different wavelength range of maximum efficiency, as it is demonstrated in Table 1.

Table 1 - Photosensitizer wavelength absorbance maxima in PBS (adapted from Wainwright 1998) [36].

Photosensitizer Type	Wavelength range absorbance maximum in buffer solution (nm)
Psoralen	300-380
Porphyrin	400-450
Acridine	400-500
Phenazine	500-550
Cyanine	500-600
Perylenequinonoid	600-650
Phenothiazinium	620-660
Phthalocyanine	660-700

Depending on its molecular structure and environment, the molecule may then lose its energy by electronic or physical processes, thus returning to the ground state, or it may undergo a transition to the triplet excited state (electron spins unpaired) which may then react further by one or both of two pathways already known as the type I and type II photoprocesses [1,36]. This gives rise to activated species which are very reactive towards the chemical environment thus producing molecular damages on important biological targets [36–38]. Typically, these compounds do not persist in the environment for long periods of time [39,40].

A photosensitizing agent with potentially optimal properties should be endowed with specific features, in addition to the expected photophysical characteristics such as a high quantum yield for the generation of both the long-lived triplet state and the cytotoxic singlet oxygen species [32].

Such features include [41–44]:

- a good absorption capacity at the wavelength of the spectral region where the light source is emitted and a good efficiency to generate ROS;
- broad spectrum of action, since one PS can act on bacteria, fungi, yeasts and parasitic protozoa;
- efficacy independent of the antibiotic resistance pattern of the given microbial strain;
- possibility to develop photodynamic protocols which lead to an extensive reduction in pathogen population with very limited damage to the host tissue;
- Cell inactivation provide a mechanism that makes minimum the risk of developing resistant strains and mutagenic processes;
- availability of formulations allowing a ready and specific delivery of the PS to the infected area;
- adequate solubility in body fluids, which affects the transport and the retention time;
- can be used in low concentrations and necessity to use of low cost light sources for activation of the photosensitizing agent;
- Be a low energetic toxic compounds and not suffer degradation by light.

In summary, the photodynamic activity of the PS to induce cell damage or death is determined by an overall lipophilicity and ionization of the photoreactive PS; quantum yield of the triplet state formation; redox potentials of the excited states (singlet and triplet) of the PS, if the reaction follows the type I pathway or the type II; the molecular extinction coefficient *e* and the quantum yield of the singlet oxygen generation, if the reaction occurs by the type II pathway [45].

A large number of different PS with photodynamic activity are now available and they are generally classified as porphyrins or non-porphyrins as described in Table 2.

Table 2 - Classification of photosensitizers as porphyrin-like or nonporphyrin-like molecules (adapted from O'Connor et al, 2009) [41].

	Compound Type	Trade name	
Photosensitizers Porphyrin-like	Hematoporphyrin	**Hematoporphyrin derivative (Photofrin®)**	**1st Generation Photosensitizers**
	Metalloporphyrins	Lutrin®	**2nd Generation Photosensitizers**
	Porphycenes		
	Pheophorbides	Tookad®	
	Purpurins		
	Clorins	NPe6 Foscan®	
	Phthalocyanines	Photosens®	
Photosensitizers Non-porphyrin-like	Psoralens		
	Anthracyclines		
	Chalcogenopyrylium dyes		
	ADPMs	ADPM derivatives	
	Cyanines	Merocyanine 540 Cationic cyanines	
	Phenothiazinium dyes	Methylene Blue Toluidine Bue Nile Blue	
Pro-drug	5-ALA	Levulan® Metvix®	

Porphyrin-derived PSs are further classified as first, second or third generation PSs. Porphyrins known from cancer photodynamic therapy were also used to kill bacteria [39].

Porphyrins are present in nature and they are essential in biochemical processes such as in oxygen transport and photosynthesis. Molecules, such as haemoglobin, myoglobin, cytochromes, chlorophylls and vitamin B, are part of the porphyrinic compounds [46]. Porphyrins are a class of aromatic heterocyclic compounds PS comprise of four pyrrole type subunits linked together by four methine bridges that forming a tetrapyrrole ring structure. Tetrapyrroles are naturally occurring pigments and one benefit of these compounds is that they don't react with other compounds and they are quenching naturally in a normal environment [20]. Accordingly, the tetrapyrrole macrocycle nucleous structure is named porphin and its derivatives are named porphyrins. Porphyrins can be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrolic macrocycle that affect the kinetics and extent of binding with microbial cells [41].

A porphyrin skeleton is essentially hydrophobic, so this factor affecting the preferential accumulation in cellular hydrophobic *loci* since such molecules must be able to get into cells by crossing lipid membranes [20,47]. Structure activity relationship studies suggest that amphiphilic derivatives (as tetracationic porphyrins) exhibit the greatest affinity for Gram (+) [26,38,48]. Some studies revealed that tetracationic porphyrins are efficient PS against both Gram (+) and Gram (-) bacteria on visible light [35,49].

First generation PSs are based on naturally porphyrins, such as hematoporphyrin (Hp) that is an endogenous porphyrin and is formed from acid hydrolysis of hemoglobin. Its derivative, hematoporphyrin derivative (HpD), gave rise to the first PS to be approved for use in PDT for treatment of cancer marketed by the name of Photofrin® [32,33].

This PS remains the most widely used and studied, but have several drawbacks, such as contamination with impurities, relatively low absorbance at 630 nm, where tissue penetration of light is not optimal, and prolonged skin photosensitivity lasting up to 6–8 weeks [41].

A number of second generation PSs have been developed to prevent certain problems associated with the first generation molecules such as prolonged skin photosensitization and suboptimal tissue penetration [37]. These PS are chemically pure compared with first

generation compounds, absorb light at a longer wavelength and cause significantly less skin photosensitization post-treatment [41].

Among the second generation of PS are the chlorins, the phthalocyanines, the metalloporphyrins and others identified in the Table 2. Chlorins are reduced porphyrins characterized by a strong enhancement of the far-red absorption band compared to metal-free porphyrins. The *meso*-tetraarylchlorin derivative 5,10,15,20-tetrakis(3-hydroxyphenyl)chlorin (mTHPC) is a hydrophilic chlorin derivative, which has the trade name Foscan® that was approved in 2001 in the European Union (EU) for the palliative treatment of head and neck cancer [1,42].

Phthalocyanines has been tested as alternatives for porphyrin and chlorin derivatives. Aluminum phthalocyanine sulfonate (AlPcS, Photosens®) is used routinely in Russia for PDT [41].

Still within the second generation are endogenous PS that are found in the body, like the protoporphyrin IX (PpIX) [31]. PpIX is an intermediate of heme synthesis. In the biosynthetic pathway of heme, the 5-aminolevulinic acid (ALA), is the biosynthetic precursor of all porphyrins in nature [50]. ALA is not a PS itself, but induces *in vivo* biosynthesis of endogenous porphyrins. ALA is normally formed from the reactants glycine and succinyl-coenzyme A in the mitochondria and is then converted into the PS-PpIX [51]. Normally, the synthesis of endogenous porphyrins is limited by the presence of free heme, but exogenous ALA bypasses this negative feedback control. The trade name of ALA is Levulan® and this compound was approved in 1999 for the treatment of actinic keratoses on the face and scalp. Studies have shown that its penetrates the skin when topically applied, however, its penetration into and nodular tumors is quite limited [50,51].

In an attempt to solve the problem of ALA hydrophilicity and hence their difficult to cell penetration, have been developed alkyl esters of ALA, the metilaminolevulinato (MAL), which presents as a trade name Metvix®. It was approved in the EU in 2001 for the topical treatment of actinic keratosis and Bowen's disease. MAL has a higher lipophilicity and are able to penetrate the cell more easily [52,53].

The third generation emerged when bound the second generation PSs with antibodies and liposomes for selective accumulation within tissue/cells/microorganisms and currently represent an active research area in the field [54].

Although the majority of PSs at the preclinical stage are porphyrin derivatives, a diverse number of non-porphyrin PSs also exist. First of all, the synthetic non-porphyrin compounds have demonstrated photosensitizing ability, like the phenothiazine dyes: methylene blue and toluidine blue [43,55]. Another group of dyes belongs to the naturally occurring PS. Psoralens (furanocoumarins) and perylenequinonoids are two examples of natural products which originally act in plants as chemical defence substances against microbial or eukaryotic organisms [45]. Significant effort is now being employed in the synthesis of pure chemical derivatives with improved activity and minimal side effects [43].

It would be desirable to have an effective PS for microbial inactivation without the need of additional chemicals, which ensures that it will bind preferentially to microbial cells instead of mammalian cells [32,52]. An important step forward in this direction was prompted by the discovery that PS that are positively charged at physiological pH values such as phenothiazines, phthalocyanines and porphyrins can directly promote the photoinactivation of both Gram (+) and Gram (-) bacteria [43,44]. Another important characteristic during the design of a PS for PDT is their water solubility where must have hydrophilic and hydrophobic characteristics, because they must be administrated in a solution, but on the other hand they must be able to cross the bacterial cell wall [56]. On the other hand, must have a positive charge being extremely important in gram-negative bacteria inactivation since their membrane structure excludes many anionic and uncharged lipophilic molecules that would lead to phototoxicity [14,35].

While phenothiazine derivatives are naturally cationic, owing to the involvement of one amino group in the π electron cloud resonance, porphyrins and phthalocyanines can be transformed into cationic entities trough the insertion of positively charged substituents in the peripheral positions of the tetrapyrrolic macrocycle (*meso* positions) and, respectively, tetraazaisoindole macrocycle, which may largely affect the kinetics and extent of binding with microbial [25,35,57].

The PS more frequently tested in microbial photoinactivation are based mainly in *meso*-substituted tetracationic porphyrins (Figure 2). The popularity of this type of PS results from their easy synthesis and potentiality toward further elaboration [43]. In fact, the synthetic approaches usually involve the condensation of pyrrole with adequate aldehydes, which are available in a wide range, providing porphyrins with different aryl or heteroaryl substituents at the *meso*-positions. Further manipulations of those substituents can give

access to a high number of porphyrins that can be designed for the desired application [40,43].

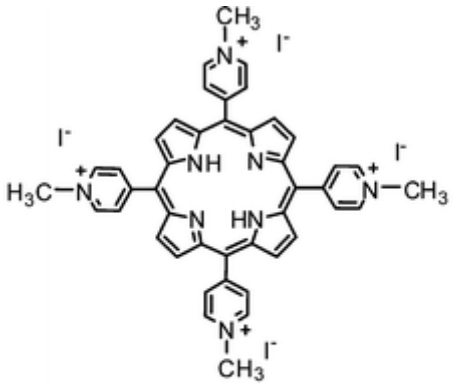


Figure 2- Example of structure of one cationic porphyrin derivative [58]

Some bacteria are also known to produce endogenous porphyrins [56]. A few studies revealed that bacteria that produce reasonable amounts of endogenous porphyrins can be efficiently degraded by photosensitization, since there is no need of break through cell barriers [59].

Therefore, porphyrins and their derivatives have been reported as one of the most promising compounds used in aPDT [46,58,60]. Porphyrins are currently used in PDT due to their unique physico-chemical properties, particularly in the treatment of superficial cancer; topical treatment of dermatological problems, such as psoriasis, acne, and Bowen's disease; gastrointestinal cancer; age related macular degeneration; cutaneous leishmaniasis; and viral infections, such as papillomatosis [42,61,62] and in the photoinactivation of microorganisms [23,43].

1.5. Antimicrobial Photodynamic Therapy (aPDT)

Antimicrobial Photodynamic Therapy (aPDT) has emerged as a potential alternative to antibiotics to treat microbial infections [28]. Membrane permeability barriers, differences

in antioxidant enzymes or DNA repair mechanisms and the size of the microbial cell are among the factors that affect the photoinactivation process. These factors are described as:

- **Bacterial target**: Microbial cells display a large variety of size, sub-cellular architecture and biochemical composition [63]. The susceptibility to photoinactivation processes can be significantly different for the various microorganisms [64]. Gram-positive bacteria have an outer wall, which is separated from the plasma membrane by a periplasmic space. Once that in their wall can be diffused macromolecules with molecular weight up to 60000 Da, and the most common PS have in generally a molecular weight of 1500 Da, these can cross the outer wall and localize in the immediate surroundings of the photosensitive endocellular sites [35]. So, the Gram (+) bacteria are relatively easy to kill by PDT, while Gram (-) bacteria show significant resistance [64]. On the contrary, the outer wall of Gram(-) bacteria possesses an additional structural element, which is external to the peptidoglycan network consisting of a glycocalyx, lipolysaccharide, outer membrane lipid bilayer, periplasm, peptidoglycan cell wall, and plasma membrane lipid bilayer [64]. This barrier keeps out most PS therefore specific methods have to be adopted to ensure that the PS can penetrate the bacterium [48].
- **Physiological state:** When the cells are in the logarithmic phase of growth are more susceptible to photodynamic inactivation than the corresponding cells in the stationary phase [63].
- **Cell density:** The cell density influence the competition for binding with the available PS, as well as for reaction with photogenerated cytotoxic species [65].

In general, the positively charged PS bound to the negatively charged surface of bacteria (Gram + and Gram −) can cause damage to bacteria by two mechanisms: DNA damage and damage to the cytoplasmic membrane, allowing inactivation of membrane transport systems and enzymes or leakage of cellular contents [66,67].

1.5.1. Advantages and disadvantages of antimicrobial photodynamic therapy

Antimicrobial photodynamic therapy as other type of therapies includes advantages and disadvantages. Below (Table 3) are described the most significant advantages/disadvantages of the treatment by aPDT.

Table 3 – Same advantages and disadvantages of aPDT [16,20,41,48,68].

Advantages	Disadvantages
✓ Non-invasive; ✓ Selective: targeted to select part of organism/tissues (does not influence on whole organism); ✓ Few side effects; ✓ Can be combined with other conventional treatments; ✓ Equally effective at killing both multi-drug resistant microbes as well as native bacterial strains; ✓ The effect of aPDT on microorganisms is much more rapid as compared to that of other antimicrobial agentes; ✓ Does not damage the tissues, in particular tissue elements such as collagen and elastin in skin; ✓ Not generating specific mechanisms of resistance.	× Difficulty to obtain the ideal PS with all of the adequate characteristics; × The lack of highly effective antimicrobial clinically approved PSs; × New PSs have not yet to be subjected to the rigorous and costly toxicological and safety studies necessary for approval for human use; × Treatment may be associated with pain; × Cutaneous photosensitivity is common; × Occasional blistering may occur and hyperpigmentation can occur at treated sites; × Located: PDT can only treat areas where light can reach. × PDT can't be used in people who have certain blood diseases, such as any of the porphyrias or people who are allergic to porphyrins.

1.5.2. Antimicrobial Photodynamic Therapy of *Staphylococcus aureus*

Staphylococcus aureus is an important human pathogen and a common etiological factor of health care associated as well as community acquired infections [69]. The ability of *S. aureus* to develop multidrug resistance is well documented, so it is crucial to find

alternative antimicrobial treatment to which this bacterium [70,71]. A potential alternative can be the aPDT to inactivate microbial cells [19,21,72,73].

S. aureus is a versatile opportunistic pathogen that is responsible for a wide variety of conditions, ranging from superficial skin infections to severe, invasive diseases [74,75]. *S. aureus* skin infections are very contagious, including the following [75–77]:

- **Folliculitis** is the least serious. A hair root (follicle) is infected, causing a slightly painful, tiny pimple at the base of a hair.
- **Impetigo** consists of shallow, fluid-filled blisters that rupture, leaving honey-colored crusts. Impetigo may itch or hurt.
- **Abscesses (boils or furuncles)** are warm, painful collections of pus just below the skin.
- **Cellulitis** is infection of skin and the tissue just under it. Cellulitis spreads, causing pain and redness.
- **Toxic epidermal necrolysis** and, in newborns, **scalded skin syndrome** are serious infections. Both lead to large-scale peeling of skin.
- **Necrotizing fasciitis** is a severe, rare, potentially lethal soft tissue infection that develops in the scrotum and perineum, the abdominal wall, or the extremities.

The use of antibiotics is yet the first treatment option to treat and sometimes to eradicate diseases caused by *S. aureus* [28,78]. An example of treatment could be the use of penicillin regardless of strain, now many of its strains are resistant to beta-lactams, macrolides, and even vancomycin, the ‘‘drug of last resort’’[70,79]. Methicillin-resistant (MRSA) and vancomycin resistant *S. aureus* (VRSA) are collectively recognized as a very serious health threat [8,80].

In vitro studies have shown that *S. aureus* is sensitive to porphyrins, phthalocyanines and others PS. Some of these studies are summarized in Table 4.

Table 4 – Summary of some studies demonstrating the effect of different PDT conditions on *S. aureus* survival *in vitro*, *ex vivo* and *in vivo*.

Article	Title	PS	Total light dose	Results
In vitro				
Bartolomeu, 2016 [81]	**Effect of Photodynamic Therapy on the Virulence Factors of *Staphylococcus aureus.***	5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) - **5.0 µM**	40 W/m^2	Reduction of 5 log CFU mL^{-1}
Almeida, 2014 [82]	**Photodynamic inactivation of multidrug-resistant bacteria in hospital wastewaters: influence of residual.**	5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) - **5.0 µM**	40 W/m^2	reduction of 4 – 5 log CFU mL^{-1}
Mai, 2016 [29]	**The Antibacterial Effect of Sinoporphyrin Sodium Photodynamic Therapy on *Staphylococcus aureus* Planktonic and Biofilm Cultures.**	Sinoporphyrin sodium (DVDMS) – **2 µM**	10 J/cm^2	≥ 90% of the bacteria were eradicated
		Sinoporphyrin sodium (DVDMS) – **5µM**	100 J/cm^2	Reduction of 4 log CFU mL^{-1}
Hsieh, 2014 [83]	**5-Aminolevulinic acid induced photodynamic inactivation on *Staphylococcus aureus* and *Pseudomonas aeruginosa***	5-aminolevulinic acid (ALA) **– 1 mM**	162 J/cm^2	90% of the bacteria were eradicated
Ex vivo				
Lambrechts, 2005 [11]	**Photodynamic therapy for *Staphylococcus aureus* infected burn wounds in mice.**	meso-mono-phenyl-tri(*N*-methyl-4-pyridyl)-porphyrin (PTMPP) (Tri-Py^+-Me-Ph)- **500 µM**	210 J/cm^2	98% of the bacteria were eradicated
Maisch, 2007 [84]	**Determination of the antibacterial efficacy of a new porphyrin-based photosensitizer against MRSA *ex vivo***	XF-73 (exeporfinium chloride) – **10 µM**	210 J/cm^2	Reduction of ≈ 3.5 log CFU mL^{-1}
In vivo				
Zolfaghari, 2009 [85]	**In vivo killing of *Staphylococcus aureus* using a light-activated antimicrobial agent**	Methylene Blue (MB) **– 100 µg/mL**	360 J/cm^2	Reduction of ≈ 1.5 log CFU mL^{-1}
Grinholc, 2015 [86]	**Antimicrobial photodynamic therapy with fulleropyrrolidine: photoinactivation mechanism of *Staphylococcus aureus*, in vitro and *in vivo* studies**	N- mthylpyrrolidimium fullerene iodide - **30 µM**	500 W/m^2	Reduction of 2 log CFU mL^{-1}

Because the delivery of visible light to living tissue is almost by definition a localized process, PDT for infections is likely to be applied exclusively to localized disease, as opposed to systemic infections such as bacteremia [11].

2. Antibiotics

Antibiotics are derived from three sources: moulds or fungi; bacteria; or synthetic or semi-synthetic compounds [87]. They can be used either internally or topically, and their function is to either inhibit the growth of pathogens or to kill them [88]. Antibiotics represents one of the most revolutionary progresses made in scientific medicine to destroy selectively microorganisms, resulting in the treatment and sometimes complete eradication of earlier incurable diseases [28,89].

It might have been supposed that at the beginning of the twenty first century, microbiologically-based diseases would have been reduced to a level that no longer had a serious impact on human health [88]. There are various antibiotics available and they come in various different brand names [87]. Antibiotics are usually grouped together based on how they work [71]. Each type of antibiotic only works against certain types of bacterium [28]. This is why different antibiotics are used to treat different types of infection [89]. Some antibiotics was referred and illustrated in the figure 3.

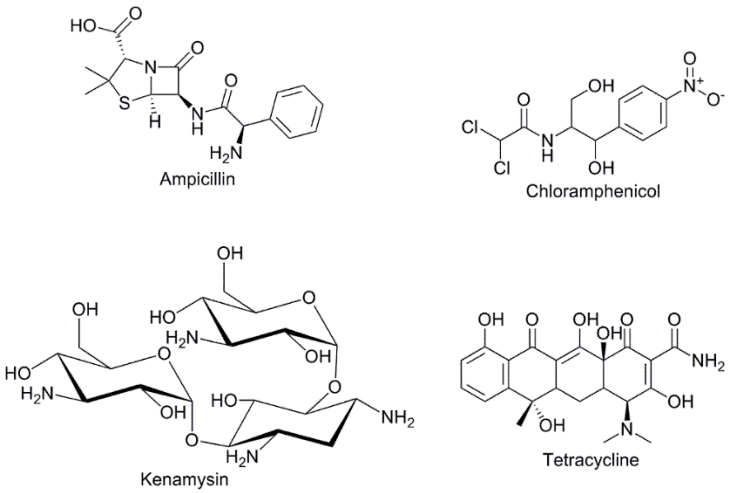


Figure 3 – Structures of some antibiotics.

2.1. Penicillin's - β-lactam antibiotics

The β-lactam antibiotics include a number of drugs with a chemical structure and a common mechanism of action. In fact, this class of antibiotics has a β-lactam ring which may be fused or linked with cyclic non-cyclic radical structures [70,71]. These antibiotics inhibit the peptidoglycan synthesis of the bacterial wall [78].The integrity of the β-lactam ring is essential for exercise the antibiotic activity. Some bacteria produce enzymes, the β-lactamases, that opening the β-lactam ring and completely nullifies the antibiotic action [90]. The penicillin's can be classified according to their antibacterial activity [70]: Natural penicillin's; aminopenicillins and others.

2.1.1. Natural penicillin's

The benzylpenicillin (penicillin G) are a natural penicillin that currently are use in clinical practice [78,91]. Currently, a growing emergency producing bacterial strains that produces β-lactamases (resistant to penicillins), led to the need for administered this antibiotic with the combination with inactivators of β-lactamases [71]. The action of benzylpenicillin on aerobic gram-positive cocci as *Staphylococcus spp.* are efficient when this specie is sensitive, because do not produce beta-lactamase enzymes [70,78]. However, the majority of clinical Staphylococcal infections are caused by strains producing β-lactamases, so that benzylpenicillin has no further interest in the treatment of such infections [70]. Nevertheless, there are still situations in which the benzylpenicillin continues to be used as a drug of choice [70].

2.1.2. Aminopenicillins

The aminopenicillins including amoxicillin, ampicillin and bacampicillin [71]. These aminopenicillins have an additional hydrophilic groups and are particularly effective against gram-negative bacteria that not produce β-lactamases [92]. However, your spectrum of action may include some gram-positive bacteria. The differences from penicillin are the presence of an amino group. That amino group helps the drug penetrate the outer membrane

of gram (-) bacterium [92]. Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell walls. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis [70].

2.2. Chloramphenicol

Chloramphenicol is an antibiotic produced synthetically, which acts by inhibition of bacterial protein synthesis. Chloramphenicol binds reversibly to the 50S ribosomal subunit and preventing binding of end of the tRNA, and therefore bacterial protein synthesis. Chloramphenicol has a very broad spectrum of action and it is mainly bacteriostatic, acts against most bacteria except the Mycobacteriaciae, Treponema and Actinomyces [93]. Furthermore, this antibiotic is ineffective against viruses, fungi and protozoa.

2.3. Aminoglycosides

Aminoglycoside antibiotics comprising a set formed of two or more amino sugars joined by a glycosidic linkage to a hexose aminated. Most of aminoglycosides have a natural origin. However, amikacin and gentamicin are two exceptions to the extent they are respectively semisynthetic derivative of sisomicin and kanamycin [94]. The kanamycin was formerly used in the treatment of severe infections caused by Gram-negative, but currently this antibiotic has low clinical use. Aminoglycosides act by interfering with protein synthesis and have a bactericidal effect (and not bacteriostatic effect, as all the other inhibitors of protein synthesis) [95]. The antibacterial spectrum of these antibiotics is quite extensive, including gram-negative bacteria, gram-positive and bacteria do not stain the Gram (such as the Koch's bacillus). In turn, the obligatory and facultative anaerobes are resistant to the action of aminoglycosides [94].

S. aureus and *epidermidis* are sensitive to aminoglycosides, whether or not produce β-lactamase. However, *Staphylococcus* quickly develop resistance against aminoglycosides.

Aminoglycosides are generally used in combination with other classes of antibiotics which have a synergistic effect between these antibiotics, especially the β-lactam [95].

2.4. Tetracycline

Tetracyclines act by blocking bacterial protein synthesis, bind to the 30S ribosome subunit, preventing the aminoacyl tRNA binding to ribosomal receptor, and thus the growth of the peptide chain [96]. The effect of tetracyclines on the bacteria is reversible, such that, upon interruption of their use, the non-return eliminated microorganisms to multiply [97].

Tetracyclines are antibiotics with broad-spectrum action, with bacteriostatic action against various gram-positive and gram-negative and bacteria that not stain the Gram (including mycoplasmas, chlamydiae, rickettsiae) and some protozoans [97,98].

Table 5 – Some of antibiotics used to treat bacterial infections by *Staphylococcus aureus* (EUCAST- European Committee on Antimicrobial Susceptibility Testing 2016) [99].

Class/Antibiotics	Mechanism of action	Effect on bacteria	Spectrum
Penicillins **Penicillin G**	Interfer with bacterial cell wall synthesis	Bactericidal	Active against non β-lactamase–producing gram (+) cocci (*Pneumococci*, *Staphylococci*, *Streptococci*), few gram (-) cocci (*meningococci* and*gonococci*), gram (+) bacilli (*Bacillus anthracis*, *Bacillus erfringens*, *Bacillus diphtheriae*) and anaerobes (*Clostridum perfringens*, *C. tetani*).
Aminopenicillins **Ampicillin**	Inhibitor of cell wall synthesis	Bactericidal	Aminopenicillins are similar to penicillin G in the activity against Gram (+) organisms but are slightly weaker than the latter. Aminopenicillins are more active against *enterococci* and *Listeria monocytogenes* compared to penicillin G. Gram (-) spectrum incudes *Haemophilus influenza*, *Salmonella*, *Shigella*, *Escherichia coli*, *Proteus mirabilis*, *N. gonorrhoeae*, *N. meningitidis*.
Chloramphenicol **Chloramphenicol**	Inhibition of bacterial protein synthesis	Bacteriostatic	Among the bacteria more sensitive are *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae*, *Salmonella typhi*, *Brucella*, *Bordetella pertussis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and anaerobic bacteria (*Clostridium* and *Bacteroides fragilis*), *Staphylococcus aureus* and some Enterobacteriaceae (such as *E. coli*, *Proteus mirabilis*, *Shigella*, *Pseudomonas pseudomallei*, *Klebsiella*, and some strains of *Enterobacter* and *Serratia*).
Aminoglycosides **Kanamycin**	Inhibition of bacterial protein synthesis	Bactericidal	Aminoglycosides have high activity against aerobic gram (-) bacilli (*E. coli*, *Proteus*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Shigella* and *Pseudomonas aeruginosa*), *Staphylococcus aureus* and *epidermidis.*
Tetracycline **Tetracycline**	Inhibition of bacterial protein synthesis	Bacteriostatic	Action against various gram (+) and gram (-) such as *Staphylococci*, *Enterococci*, P*neumococci*, *Gonococci*, some *Enterobacteriaceae* (such as *Salmonella* and *Shigella*), bacteria that not stain the Gram (including mycoplasmas, chlamydiae, rickettsiae) and some protozoans.

2.5. Microbial resistance

The rapidly increasing emergence of antibiotic resistance amongst pathogenic bacteria assume increasing economic and social impact due to the high morbidity and mortality induced by the proliferation of antimicrobial resistance [78,80]. Bacteria replicate very rapidly and a mutation that helps a microbe to survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population [28,100]. The inappropriate prescription of antibiotics and the failure of some patients to complete their treatment regimen also exacerbate the problem [101].

The intended modes of action of antibiotics may be counter-acted by bacterial organisms via several different means [102]. This may involve preventing antibiotic access into the bacterial cell or perhaps removal or even degradation of the active component of the antimicrobial agent [78,103]. In fact, several different mechanisms may work together to confer resistance to a single antimicrobial agent [88]. Some of this mechanisms are described below:

- **Intrinsic Resistance**

Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class [88,104].

- **Acquired Resistance**

Acquired resistance is said to occur when a particular microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible [89,105]. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes through a small circular DNA (plasmids) or from a combination of these two mechanisms [103,106].

Acquired resistance results from successful gene change and/or exchange that may involve: mutation or horizontal gene transfer via transformation, transduction or conjugation [89,102] described in Table 6.

Table 6 – Mechanisms that envolve the acquired resistance.

Mechanism	Description
Transformation	Involves uptake of short fragments of naked DNA by naturally transformable bacteria [103].
Transduction	Involves transfer of DNA from one bacterium into another via bacteriophages [107].
Conjugation	Involves transfer of DNA via sexual pilus and requires cell–to-cell contact. DNA fragments that contain resistance genes from resistant donors can then make previously susceptible bacteria express resistance as coded by these newly acquired resistance genes [89,102].

- **Mutation**

A mutation is a spontaneous change in the DNA sequence within the gene that may lead to a change in the trait which it codes for [104,106]. Any change in a single base pair may lead to a corresponding change in one or more of the amino acids for which it codes, which can then change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials [102,105].

CHAPTER 2

Combining Antibiotics and Photodynamic Therapy to Inactivate *Staphylococcus aureus* on skin

1. Introduction

Staphylococcus aureus belongs to the natural microflora, resides on the surface of the skin and on mucous membranes of warm-blooded animals (present in the nose of about 30% of healthy adults and on the skin of about 20%, and at higher percentages in patients admitted to hospitals and people who work there), but it may become pathogenic in conditions such as breakage of the skin barrier or decreased immunity [74,108–110]. Is a common Gram-positive bacteria that is responsible for a wide variety of disorders, causing wound infections and colonize the mucous of nasal cavity and normal skin of healthy population [110,111].

Its pathogenesis is dependent on the secretion of an array of virulence factors, the surface exposure of multiple cell wall anchored proteins [74] and extracellular components that are expressed during the different stages of infection: colonization, avoidance or invasion of the host immune defense, growth and cellular division culminating in bacterial dissemination, causing toxic effects to the host [112,113].

S. aureus infections are common in skin, but the bacterium can spread through the bloodstream and infect distant organs. As the bacterium tends to infect the skin, often causing abscesses [79], but as the bacterium can travel through the bloodstream, causing bacteremia and infect almost any site in the body, particularly heart valves (endocarditis) and bones (osteomyelitis) [76,114]. The bacterium also tend to accumulate on medical devices in the body, such as heart pacemakers, and catheters inserted through the skin into blood vessels [76].

These infections are usually treated with antibiotics that are chosen based on whether they are likely to be effective against the strain causing the infection. The penicillin, aminopenicillins, cephalosporin, aminoglycoside, tetracyclines and chloramphenicol are effective antibiotics to combat infection by *S. aureus*. However, both community-associated and hospital-acquired infections with *S. aureus* have increased in the past three decades, and

the rise in incidence has been accompanied by a rise in antibiotic-resistant strains [24,101], particularly methicillin-resistant *S aureus* (MRSA) and vancomycin-resistant strains (VRSA) [115,116] which are collectively recognized as a very serious health threat [8,80]. The resistance to penicillin emerged in the mid-1940s, only a few years after the introduction of this antibiotic in the clinical practice [101]. Later, in 1959, the semi-synthetic antibiotic methicillin was introduced for the treatment of infections caused by penicillin-resistant *S. aureus* [80]. Yet, in 1961 the first cases of methicillin- resistant *S. aureus* (MRSA) isolates [24] were reported and currently, only few compounds are still effective in the treatment of MRSA infections [101]. Vancomycin, the ‘‘drug of last resort’’has been the most reliable therapeutic agent against infections caused by meticillin-resistant *S. aureus* (MRSA). However, in 1996 the first MRSA to acquire resistance to vancomycin, was isolated from a Japanese patient. Subsequent isolation of several vancomycin resistant S. aureus (VRSA) strains from USA, France, Korea, South Africa, and Brazil has confirmed that emergence of vancomycin resistance in *S. aureus* is a global issue [70,79]. This resistance comes from the bacterial gene mutation and horizontal transfer of resistance genes from external sources [117,118].

Although the intensive overuse of antimicrobial drugs began to exert new survival pressure on relevant microorganisms, antibacterial resistance was hardly acknowledged in the past as novel antibiotics became steadily available and were readily modified and improved for clinical use [24,101,117]. The development of novel but still conventional antibiotics, is not likely to solve the problem as it is probably only a matter of time until they will also ineffective. Bacterial resistance is undoubtedly recognized as a major medical challenge in most healthcare systems. Consequently, the traditional treatment of bacterial infections with antibiotics can be difficult, emphasizing the importance and urgency to develop new alternative treatments to treat bacterial infections. In this sense, new alternative therapies such as antimicrobial photodynamic therapy (aPDT) have been proposed.

aPDT is being actively studied as a possible alternative to antibiotic treatment as in localized infections [60,64,119]. aPDT involves the combination of light, oxygen and PS that trust be able to produce ROS upon irradiation with light. Thus, when the dye absorbs a photon, an electron is promoted from its ground state to an electronically excited state that returns the energy. Although originally employed in the treatment of cancer during the last decade, an increasing number of studies on PDT application have been published about

microbiocidal effect in addition to better access to sites that are inaccessible to conventional therapy [120]. This technology has already shown to be effective against Gram-positive and Gram-negative bacteria, viruses, fungi, and parasites [64,119,121]. The major advantage of this technology over antibiotics is the multi-target action [122] and no emergence of resistances [28,81,123].

Although it is well known that the use of large amounts of antibiotics in clinical practice is undesirable, since they give rise to the selection of antibiotic-resistant strains, little effort has been made to employ aPDT in order to increase the efficacy of such antibiotics, as well as to employ antibiotics to potentiate the efficacy of aPDT. Xing et al. (2011) tested a vancomycin-porphyrin conjugate to inactivate vancomycin-resistant enterococci, showing strong PDI activity of the conjugate against vancomycin-sensitive and resistant strains, when compared to vancomycin and porphyrin alone [124]. The combination of vancomycin with PDI was also useful in the disruption of *S. aureus* biofilms. Pre-treating the biofilms with PDI and then apply vancomycin at concentrations bellow the biofilm inhibitory concentration, causes a disintegration of the biofilm matrix and allows the killing of bacteria almost entirely [27]. Malik and Nitzan (1995) tested also the combination of different natural porphyrin derivatives and antibiotics (methicillin, ampicillin, polymyxin B nonapeptide, tetracycline) to inactivate both multi-resistant Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) [125]. The photoinactivation of four multidrug resistant bacteria by a porphyrin derivative in the presence of ampicillin and chloramphenicol was also evaluated and the results showed that in the presence of porphyrins and antibiotics the bacterial photoinactivation was higher than when the porphyrin derivatives were used alone [49].

The aim of this study was to evaluate the antimicrobial applicability of aPDT to treat skin infections by *S. aureus*. Besides, the synergism of the combination of this therapy with conventional antibiotics was also studied.

2. Methods

2.1. Photosensitizer

The PS applied in this study was a tetracationic porphyrin, the 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^{+}-Me) and was synthetized according to previously described procedure [126]. A stock solution of this porphyrin, at 500 μM, was prepared in dimethyl sulfoxide and stored in the dark. Before each assay the porphyrin solution was sonicated during 30 min at room temperature (ultrasonic bath 0.6L | Nahita).

2.2. Bacterial strains and growth conditions

The strain of *S. aureus* (ATCC 6538) was inoculated on solid medium BD Baird-Parker Agar (BPA)(Liofilchem) at 37 ºC during 24 h and posteriorly kept a 4 ºC (Work Sample). The bacterium was inoculated whenever necessary in liquid medium Brain-Heart Infusion (BHI) (Liofilchem) and grown aerobically at 37 ºC for 24 h under stirring (130 rpm). For each assay, an aliquot of this culture (300 μL) was transferred twice into a new fresh BHI medium (subcultured in 30 mL) and grew overnight at 37 °C under stirring.

2.3. Irradiation conditions

Following the dark incubation period (10 min for in vitro and 30 min for *ex vivo*), samples were exposed, in parallel, to white light: PAR radiation (13 OSRAM 21 lamps of 18 W each, 380-700 nm) with an irradiance of 40 W m^{-2} or Lumacare system (an interchangeable fiber optic probe - 400–800 nm - coupled to a 250 W quartz/halogen lamp (LC-122; LumaCare, Newport Beach, CA, USA) with an irradiance of 150 W m^{-2}, at 25 °C for 180 - 270 min.

2.4. Photoinactivation assays in PBS

Overnight bacterial cultures, were tenfold diluted in phosphate-buffered saline (PBS) to a final concentration of ~10^{7-8} CFU mL^{-1} (colony-forming unit per mililitre). The diluted bacterial suspensions were distributed in 50 mL beakers (final volume of 10 mL per beaker) and incubated in the dark for 10 min at room temperature under 100 rpm stirring with 5.0 μM of porphyrin (Tetra-Py^{+}-Me), to promote the PS binding to cells. Then the beakers are

irradiated by white light with an irradiance of 40 W m^{-2}. Bacterial suspensions were irradiated up to 270 min (total light dose of 64.8 J cm^{-2}) and sub-samples of 100 µL were collected at the beginning of the irradiation (time 0) and after 15, 30, 60, 90, 180 and 270 min of exposure to light. After each photosensitization period, the cells were serially diluted in PBS, pour-plated in solid medium trypticase soy agar (TSA) (Liofilchem) and incubated at 37 °C for 24 h, for viability monitoring. The cell viability was determined by counting the CFU mL^{-1} on the most appropriate dilution. Control samples were included in all PDI experiments: light control (LC) consisted of a bacterial suspension that was exposed to light; and dark control (DC) consisted of a bacterial suspension incubated with PS at the maximum concentration under the same conditions as the samples, but protected from light. Three independent experiments were conducted.

2.5. Photoinactivation assays in PBS combined with antibiotics

In these assays, the photoinactivation of *S. aureus* with the PS at 5.0 µM in the presence of five antibiotics: ampicillin (Applichen Panreae), chloramphenicol (Applichen Panreae), Kanamycin (Applichen Panreae), Penicillin G (Sigma Life-Science) and Tetracycline (Sigma Life-Science) was evaluated. All the antibiotics were tested at the minimum inhibitory concentrations (MIC) according EUCAST- European Committee on Antimicrobial Susceptibility Testing (2016) and accordingly to the Clinical and Laboratory Standards Institute [CLSI] (2013) [99,127]. The MIC concentrations were, 0.25 µg mL^{-1}, 8 µg mL^{-1}, 2 µg mL^{-1}, 0.125 µg mL^{-1} and 1.0 µg mL^{-1}, respectively, for ampicillin, chloramphenicol, kanamycin, penicillin G and tetracycline. For ampicillin two more concentrations were tested 0.5 µg mL^{-1} and 1.0 µg mL^{-1}.

For each assay four goblets of 50 mL were prepared: one with bacteria diluted 1:10 in PBS and PS (Sample); other with bacteria diluted 1:10 in PBS and PS, plus antibiotic (PS + (abbreviation of the used antibiotic)); other with bacteria diluted 1:10 in PBS and antibiotic (Antibiotic Control - AC); other with bacteria diluted 1:10 in PBS and PS, which was kept in dark conditions, wrapped in aluminium foils (DC) and other with bacteria diluted 1:10 in PBS (LC). The quantity of antibiotic was the same used in the Sample and in the Antibiotic Control. The goblets were kept under irradiation 180 min and aliquots were taken at 0, 15, 30, 60, 90 and 180 min. The aliquots were diluted in PBS and plated in TSA by

incorporation. After 24 h at 37 ºC, the number of colonies was enumerated and the results were expressed in CFU mL^{-1}. For each assay was done three independent experiments in duplicates.

Before aPDT assays the susceptibility of the *S. aureus* strain to the used antibiotics was evaluated using the Disk Diffusion Test. Overnight bacterial cultures in liquid medium BHI, growth at 37 °C, was diluted 1:100 in saline solution until a turbidity compatible with the 0.5 standard of MacFarland ($1x10^6$ CFU/mL) [128]. With a sterile swab was seeded on the surface of Mueller Hinton agar (MHA) in Petri plates. Then the discs were placed on the plates which were incubated posteriorly at 37 °C for 24 h. The sensitivity was determined by measuring the halo of growth inhibition diameter and compared with the zone diameter breakpoint established by EUCAST- European Committee on Antimicrobial Susceptibility Testing (2016) and accordingly to the Clinical and Laboratory Standards Institute [CLSI] (2013) [99,127].

2.6. Photoinactivation assays in skin model (*ex vivo*)

Its performed assays of aPDT in pocine skin with a porphyrin (Tetra-Py^+-Me) in different conditions.

2.6.1. Preparation of porcine skin

Fresh skin of porcine was obtained from a local gash. The skin was cut into four portions of area 10 cm^2 (5 x 2 cm) pieces (one of them used as test sample and three as controls) and the adipose tissue beneath the dermis was removed with a scalpel. The excised skin was placed in a sterile Petri dish and disinfected with 70% ethanol, washed with sterile PBS and placed under ultraviolet radiation for 30 min.

After, an aliquot of 500 μL of an overnight culture of *S. aureus* diluted in PBS (1:10) was distributed over the three skin pieces using an aerosol spray to obtain a density of approximately 10^5 CFU per cm^2. An aliquot of 500 μL of PS diluted in PBS (final concentration 25 μM, 40 μM or 50 μM) was sprayed on two of the skin portions (Sample and DC). The other skin portion (LC) was sprayed with 500 μL of PBS to ensure the same volume sprayed into the others skin pieces. The fourth skin portion (Bacterial Control - BC) was not contaminated with *S. aureus*, because was used as control to verify the efficiency of

the skin disinfecting to inactivate resident bacteria present in the skin, and then sprayed with 1000 μL of PBS to ensure the same volume condition.

All skin portions were covered with aluminum paper and incubated for 30 min in dark, to promote the PS binding to skin cells. The porcine skin samples were irradiated with two different white light conditions with irradiances of 40 W m^{-2} and 150 W m^{-2}. The dark control must be covered with aluminum paper. After irradiation, a sterile cotton wool swab was used to remove the bacteria from each skin portion after 0, 60, 90, 120 and 180 min. The cotton wool swab was moistened in PBS before swabbing the skin surface. The cotton wool swab was passed on each skin piece 30 times. The bacteria present in the cotton wool swab were suspended in 2.0 mL of PBS diluted in PBS, pour-plated in solid medium TSA. The plates were incubated at 37 °C for 24 h, for viability monitoring. The cell viability was determined by counting the CFU on the most appropriate dilution. Three independent experiments were conducted in duplicate.

2.7. Photoinactivation assays combined with antibiotic in the skin model (*ex vivo*)

The same previously described protocol, including two more samples: antibiotic control (AC) and PS with antibiotic (PS+antibiotic), was used. In these experiments, only ampicillin was used at 1.0 μg mL^{-1} and 5 μg mL^{-1}. The PS was tested at 40 μM or 50 μM under white light irradiance of 150 W m^{-2}.

2.8. Statistical Analysis

Statistical analyses were performed using GraphPad® Prism 6.01. Normal distributions were assessed by the same software. The significance of a Tetra-Py^{+}-Me and irradiation time on bacterial was assessed by two-way ANOVA analysis of variance. Morover, when the significance was accepted, at ($p < 0.05$), Tukey´s multiple comparison test was used for a pairwise comparison of the means. Three independent experiments were conducted in duplicate for each assay.

3. Results

The clinical strain employed in this study, the *S. aureus* (ATCC 6538) is susceptible to all tested antibiotics (Figue 4), ampicillin, chloramphenicol, kanamycin, penicillin G and tetracycline.

Antibiotics	Standards zone diameter breakpoint (mm)		Results of diameter breakpoint (mm)	Images
	S ≥	R <		
Ampicillin	29	28	32	
Penicillin G	26	26	40	
Kanamycin	18	13	26	
Chloramphenicol	18	18	20	
Tetracycline	22	19	21	

Figure 4 – Drug-resistance profile to antibiotics for the studied strain.

3.1. Photoinactivation of bacteria in PBS

The aPDT with 5.0 µM of Tetra-Py^{+}-Me and an irradiance of 40 W m^{-2} was effective against *S. aureus* in PBS, leading to a reduction in colony forming units (CFU) of 8.0 log, after 180 min of irradiation (Figue 5). Light controls and dark controls results show that the viability of *S. aureus* is not affected by irradiation itself nor by the PS used in the dark at the concentration of 5.0 µM (7-8 log CFU/mL maintained during all irradiation period) (ANOVA, $p > 0.05$).

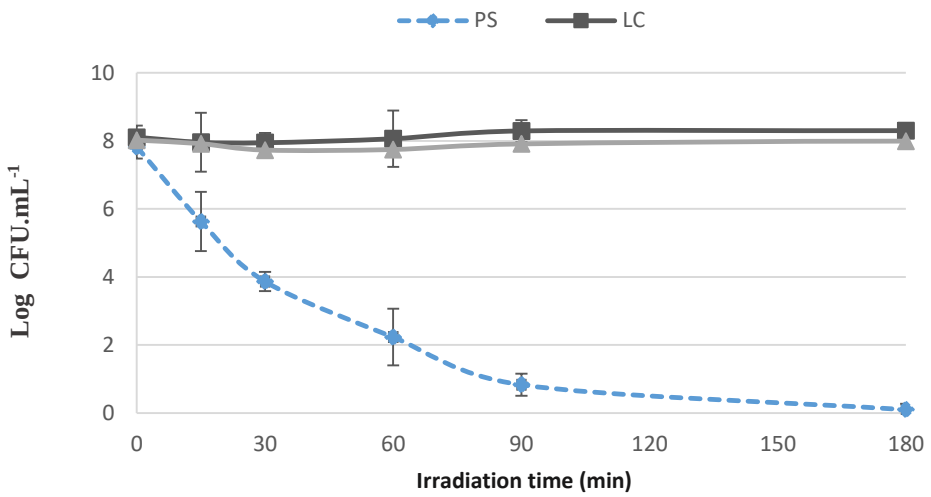


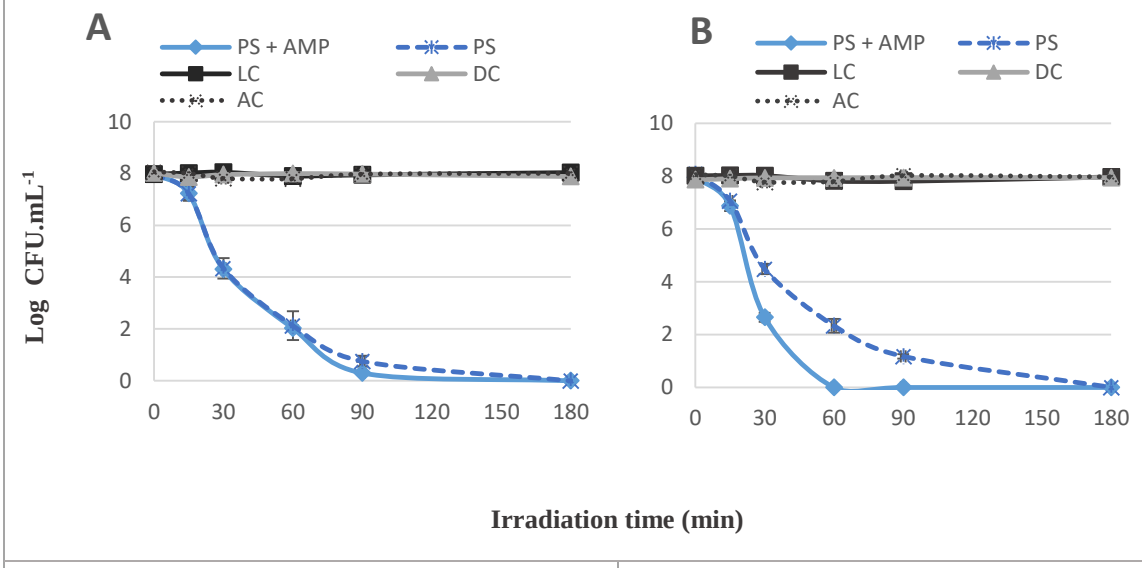
Figure 5 - Photoinactivation of *S. aureus* (ATCC 6538) in PBS, after 30, 60, 90 and 180 min, incubated with Tetra-Py^{+}-Me at 5.0 µM and irradiated with white light at an irradiance of 40 W m^{-2} (PS, Photosensitizer; LC, Light Control; DC, Dark Control). *Three independent assays were done. Each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.*

3.2. Photoinactivation of bacteria by aPDT and antibiotics in PBS

When the aPDT assays were performed in the presence of antibiotic, changes on the incativatiom profile was observed. Thus, when the ampicillin was added to the bacterial suspension and the aPDT treatment performed an increase in the efficiency of inactivation was observed for the two highest ampicillin concentrations (0.5 µM and 1.0 µM). The photoinactivation with 5.0 µM of Tetra-Py^{+}-Me in combination with ampicillin at MIC concentration (0.25 µg mL^{-1}) showed the same bacterial reduction (ANOVA, $p > 0.05$) observed when only PS was used (Figure 6 A).

When the concentration of ampicillin was increase to 0.5 µg mL^{-1} (Figure 6 B) the photoinactivation occurred earlier, showing a reduction of ~8 log after 60 min of irradiation. When ampicillin was used at 1.0 µg mL^{-1}, the aPDT efficiency increase still observed, either relatively to *S. aureus* photoinactivation without the presence of antibiotic nor to the other two antibiotic tested concentrations (ANOVA, $p < 0.05$). A reduction of ~ 8 log was achieved after 30 min of irradiation (Figure 6 C). The antibiotic controls at different concentrations of ampicillin (0.25 µg mL^{-1}, 0.5 µg mL^{-1} and 1.0 µg mL^{-1}) showed that the viability of *S. aureus*

was not affected by its presence (7 - 8 log CFU mL^{-1}) maintained the cell viability during all irradiation period (ANOVA, $p > 0.05$).



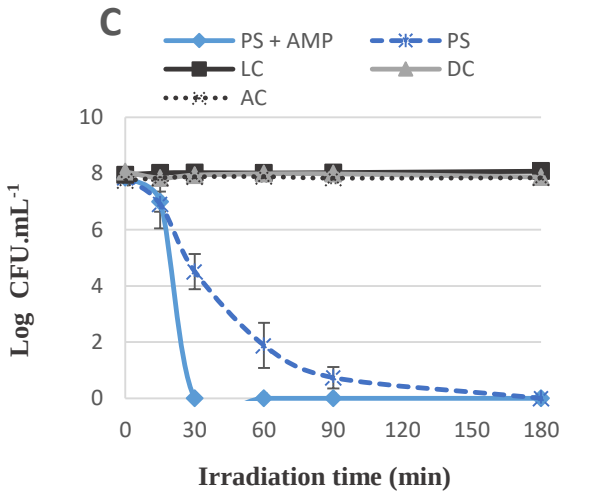


Figure 6 - Photoinactivation of *S. aureus* in PBS, after 30, 60, 90 and 180 min of irradiation at 40 W m^{-2}, at 5.0 µM of Tetra-Py^{+}-Me and ampicillin concentrations of 0.25 µg mL^{-1} (A), 0.5 µg mL^{-1} (B), 1.0 µg mL^{-1} (C) (PS, Photosensitizer; PS + Amp, Photosensitizer with ampicillin; LC, Light Control; DC, Dark Control; AC, ampicillin and light). *Three independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.*

The aPDT treatment with Tetra-Py^{+}-Me at 5.0 µM combined with chloramphenicol at 8.0 µg mL^{-1}, point out an efficiency decrease of bacterial inactivation (1.7 log) when compared with the photoinactivation without the presence of chloramphenicol in the early inactivation times (after 60 min, ANOVA, $p < 0.05$), but for the others tested periods the *S. aureus* photoinactivation was similar in both samples conditions (ANOVA, $p > 0.05$) (Figure

7 A). The chloramphenicol alone (AC) (Figure 7 A) does not reduce the *S. aureus* abundance, which it was kept constant throughout the assay (ANOVA, $p > 0.05$).

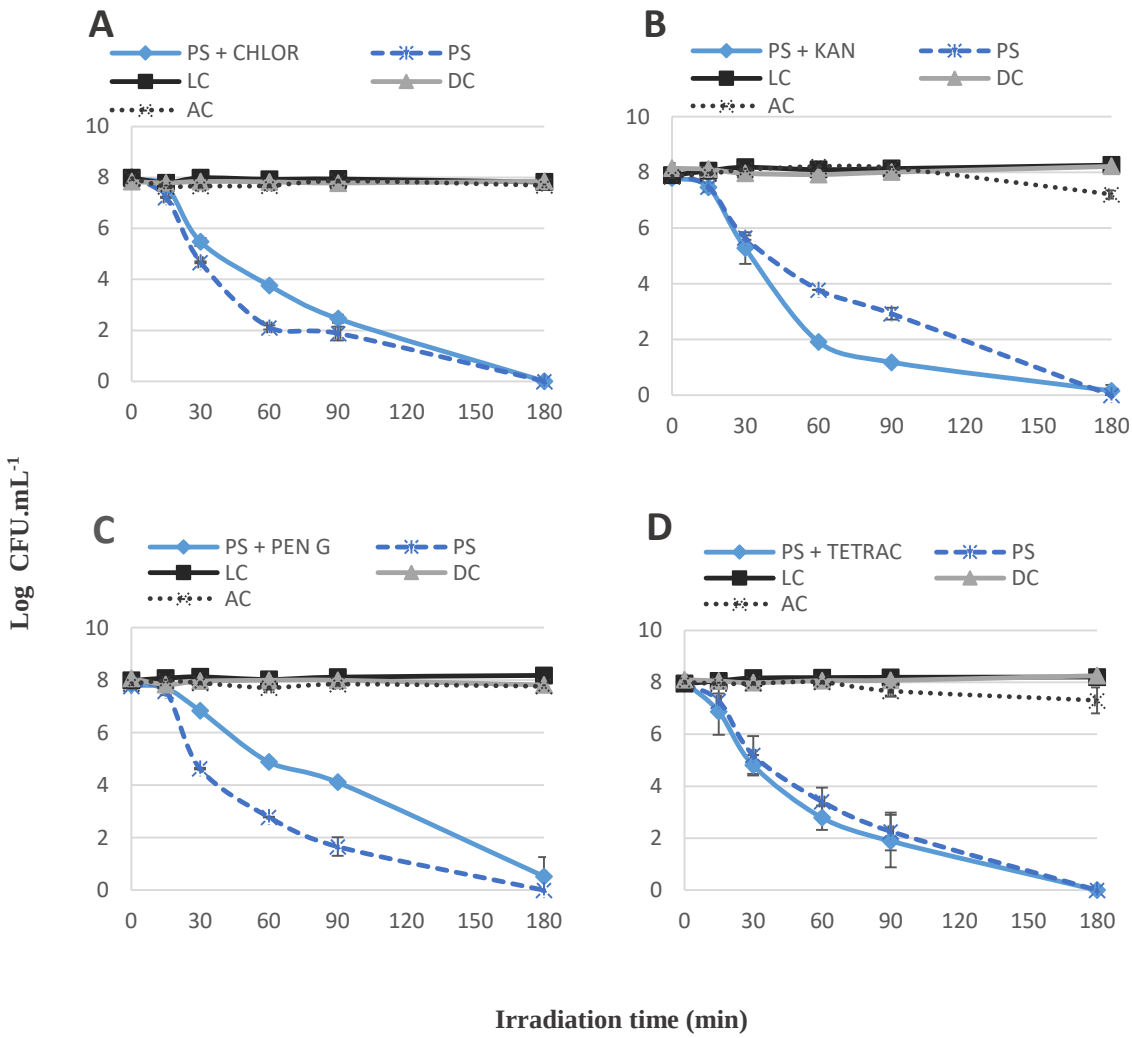


Figure 7 - Photoinactivation of *S. aureus* in PBS, after 30, 60, 90 and 180 min of irradiation at 40 W m^{-2} with a concentration 5.0 µM of Tetra-Py^{+}-Me and chloramphenicol concentrations of 8.0 µg mL^{-1} (A), Kanamycin 2.0 µg mL^{-1} (B), Penicillin G 0.125 µg mL^{-1} (C) and Tetracycline 1.0 µg mL^{-1} (D) (PS, Photosensitizer; PS + CHLOR, Photosensitizer with Chloramphenicol; PS + KAN, Photosensitizer with Kanamycin; PS + PEN G, Photosensitizer with Penicillin G; PS + TETRAC, Photosensitizer with Tetracycline; LC, Light Control; DC, Dark Control; AC, control of the antibiotic-different in each graph). *Three independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.*

For aPDT treatment associated with kanamycin at 2 μg mL^{-1} (Figure 7 B), the bacterial reduction after 60 min and 90 min of irradiation was more effective (~1.9 log) than that observed when aPDT was done without antibiotic (ANOVA, $p < 0.05$). However, after 180 min the two samples reached the same bacterial inactivation (8 log) (ANOVA, $p > 0.05$). A small reduction of 0.6 log in bacterial concentration was observed after 180 min relatively to the bacterial control (ANOVA, $p<0.05$). The aPDT treatment in the presence of penicillin G at 0.125 μg mL^{-1} showed significant difference (ANOVA, $p < 0.05$) relatively to the inactivation without antibiotic (Figure 7 C) after 30 min of irradiaton (~2 log), although the results remained similar in both samples at longer times (ANOVA, $p > 0.05$). The penicillin G alone does not affect the the *S. aureus* abundance during the assay, the concentration remained constant in all assay (7-8 log) (ANOVA, $p> 0.05$).

No significant differences were observed between aPDT in combination with tetracycline at 1.0 μg mL^{-1} and aPDT alone (Figure 7 D) (ANOVA, $p > 0.05$). The tetracycline alone reduced *S. aureus* by ~0.7 log relatively to the bacterial control after 180 min. (ANOVA, $p > 0.05$).

3.3. Influence of light source on photoinactivation on porcine skin

Both white lights, 40 W m^{-2} and 150 W m^{-2}, were effective to inactivate the bacterium, but light with the irradiance of 150 W m^{-2} causeda higher reduction. Also a significant difference between PS concentrations (25 μM and 50 μM) was observe under both light sources.

Tetra-Py^{+}-Me at 25 μM under irradiation at 40 W m^{-2} does not inactivate *S. aureus,* the concentration remained constant, about 5 log during the 180 min of irradiation (Figure 8 A). However, increasing the concentration to 50 μM, a reduction of 0.8 log was observed after 90 min (Figure 8 A). The differences between the two concentrations were significant after 60 min of irradiation (ANOVA, $p < 0.05$). No statistical difference for DC and LC was observed during the assay (ANOVA, $p > 0.05$).

When irradiation was done at an irradiance of 150 W m^{-2} (Figure 8 B) at 25 μM of Tetra-Py^{+}-Me, a reduction of *S. aureus* of about ~ 0.5 log was observed after 180 min of irradiation, but the difference relatively to the bacterial control was not significant (ANOVA, $p > 0.05$).

Increasing the concentration of Tetra-Py^{+}-Me to 50 µM and using an irradiance of 150 W m^{-2}, a decrease of about 4 log was observed after 180 min of irradiation (Figure 8 B). In this condition, bacterial concentrations were significantly different from that observed in the bacterial control afterafter 30 min until the end (ANOVA, $p < 0.05$). Light and dark controls showed no significant differences inthe viability of *S. aureus* (~5 log CFU/mL maintained during all irradiation period) (ANOVA, $p > 0.05$) (Figure 8 B).

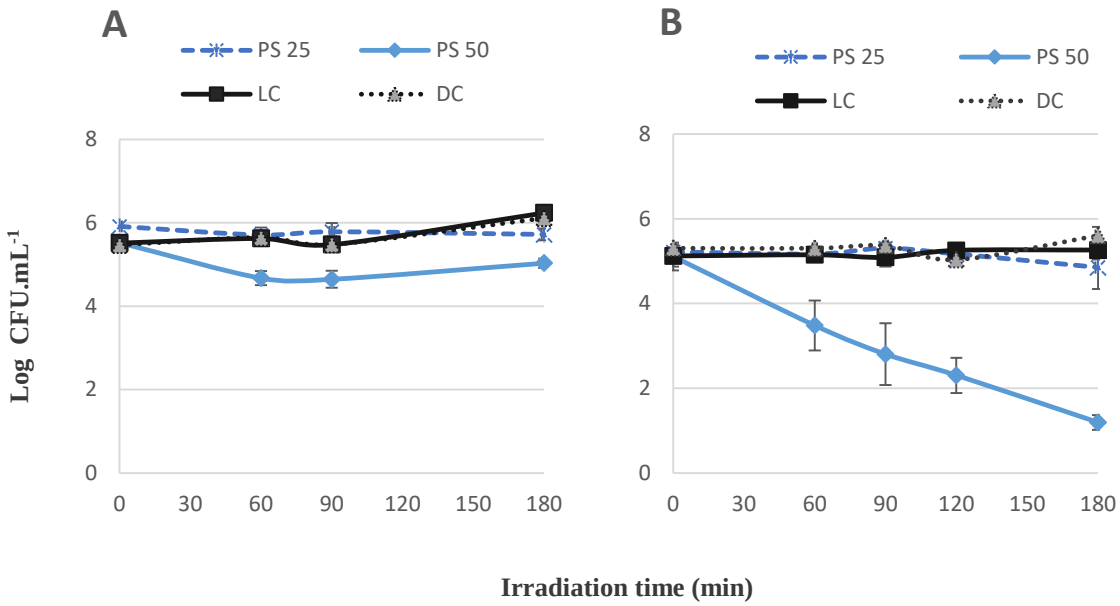


Figure 8 – (A) Photoinactivation of *S. aureus* on porcine skin, after 60, 90 and 180 min of irradiation at 40 W m^{-2} (PS 25, Photosensitizer with Tetra-Py^{+}-Me in 25 µM; PS 50, Photosensitizer with Tetra-Py^{+}-Me in 50 µM; LC, Light Control; DC, Dark Control)**. (B) Photoinactivation of *S. aureus* on porcine skin, after 60, 90, 120 and 180 min of irradiation at 150 W m^{-2}** (PS 25, Photosensitizer with Tetra-Py^{+}-Me in 25 µM; PS 50, Photosensitizer with Tetra-Py^{+}-Me in 50 µM; LC, Light Control; DC, Dark Control). *Three independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.*

3.4. Photoinactivation of bacteria by aPDT and antibiotic in porcine skin

The combination of aPDT and ampicillin on porcine skin was tested under 150 W m^{-2} with 50 µM or 40 µM of PS and 1.0 µg mL^{-1} and 5 µg mL^{-1} of antibiotic.

The photoinactivation at 50 µM of Tetra-Py^{+}-Me in the presence of ampicillin at 5 µg mL^{-1}) showing a reduction of ~5.6 log after 180 min of irradiation (Figure 9). When the

antibiotic was not added after at 180 min of irradiation, a reduction of ~4 log was observed. In the conditions of this antibiotic concentration, a reduction of ~1 log of *S. aureus* was observed after 120 min. The viability of *S. aureus* in light and dark controls was not affected (ANOVA, $p > 0.05$) (Figure 9).

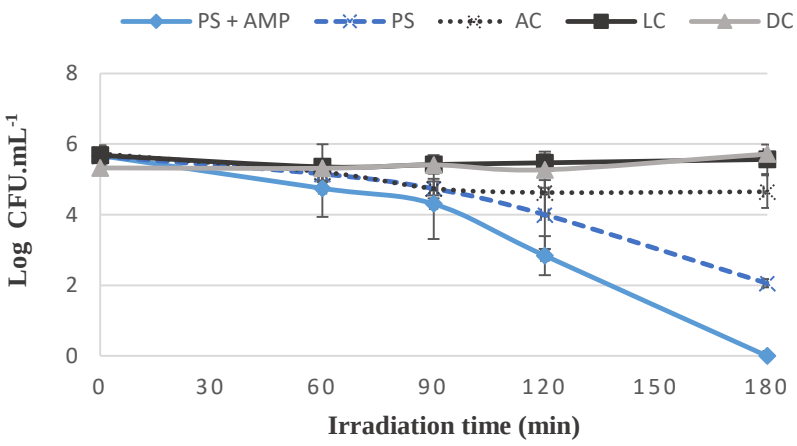


Figure 9 - Photoinactivation of *S. aureus* on porcine skin, after 60, 90, 120 and 180 min of irradiation at 150 W m^{-2} with a concentration 50 µM of Tetra-Py^{+}-Me and ampicillin in concentrations of 5 µg mL^{-1} (PS, Photosensitizer; PS + AMP, Photosensitizer with Ampicillin; LC, Light Control; DC, Dark Control). *Two independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations*

Reducing the concentration of Tetra-Py^{+}-Me to 40 µM in the presence of ampicillin at 1 µg mL^{-1} the bacterial reduction observed was similar to that observed when the antibiotic was not added (ANOVA, $p > 0.05$) (Figure 10 A). After at 180 min of irradiation, a reduction of ~ 99,99% (4 log) was observed for both aPDT and aPDT plus ampicillin (1.0 µg mL^{-1}). Only the antibiotic at this concentration, reduced ~0.5 log of *S. aureus* was observed after 180 min. The viability of *S. aureus* in light and dark controls was not affected (ANOVA, $p > 0.05$) (Figure 10 A).

Increasing the concentration of ampicillin to 5 µg mL^{-1}, in same conditions, the inactivation was also similar to that of aPDT without antibiotic addition (ANOVA, $p > 0.05$) (Figure 10 B). A reduction of of 4 log was observed for both samples after 180 min of irradiation (ANOVA, $p > 0.05$). Nevertheless, at this antibiotic concentration, a reduction of ~1 log of *S. aureus* was observed after 120 min. Bacterial concentration in both light and

dark controls was constant during all the irradiation period (ANOVA, $p > 0.05$) (Figure 10 B).

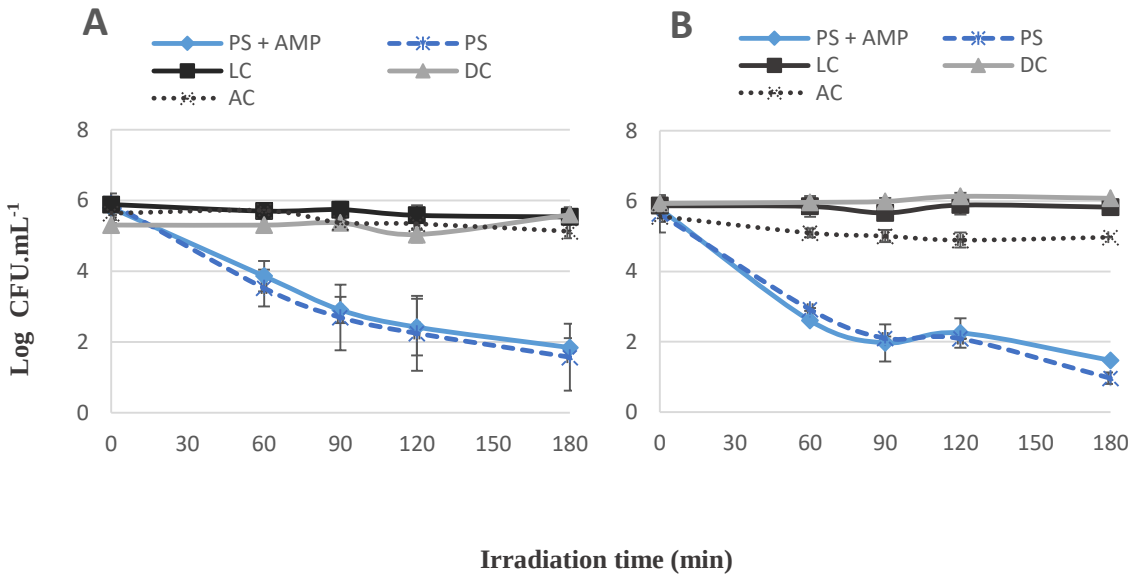


Figure 10 - Photoinactivation of *S. aureus* on porcine skin, after 60, 90, 120 and 180 min of irradiation at 150 W m^{-2} with a concentration 40 µM of Tetra-Py^{+}-Me and ampicillin in concentrations of 1.0 µg mL^{-1} (A), Ampicillin 5 µg mL^{-1} (B) (PS, Photosensitizer; PS + AMP, Photosensitizer with Ampicillin; LC, Light Control; DC, Dark Control). *Three independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations*

3.5. Photoinactivation of bacteria by aPDT in porcine skin after three cycles of treatment

When three aPDT cycles of 180 min were applied with 50 µM of porphyrin under 150 W m^{-2} in porcine skin, *S. aureus* was inactivated by 5.5 log, to the detection limit of the method. Bacterial concentration in light control was constant during all the irradiation period (ANOVA, $p > 0.05$) (Figure 11).

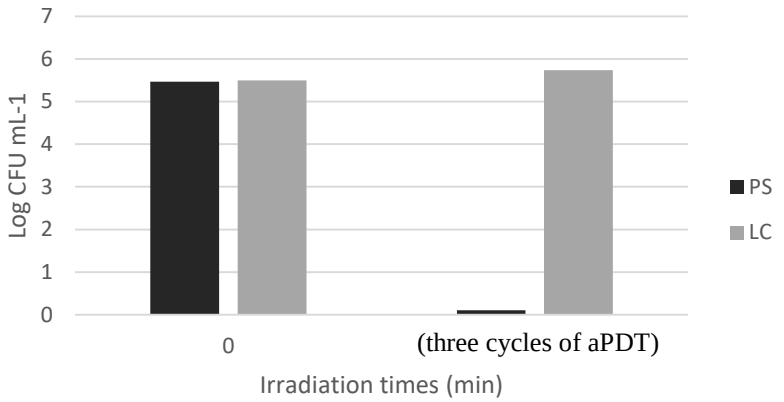


Figure 11- Photoinactivation of *S. aureus* on porcine skin, after three successive cycles of aPDT of 180 min with an irradiation at 150 W m^{-2} with a concentration 50 µM of Tetra-Py^{+}-Me (PS, Photosensitizer; LC, Light Control. *Two independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.*

3.6. Images of skin before and after treatment with aPDT

At the naked eye, no negative effects on the porcine skin were observed after 180 min of aPDT treatment with 50 µM of porphyrin under 150 W m^{-2}.

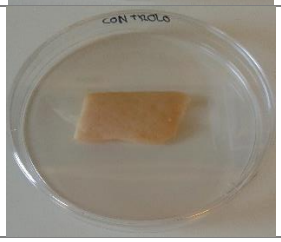
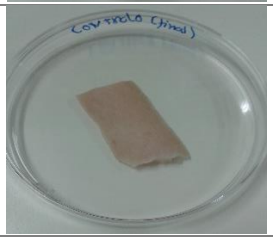
	Before treatment	After treatment
PS		
LC		

Figure 12 – Images of porcine skin before and after treatment with aPDT. (PS, Photosensitizer; LC, Light Control).

4. Discussion

Regardless of the abundant literature about *in vitro* studies of aPDT against *S. aureus* as well as about *in vivo* assays of aPDT to treat *S. aureus* wound infections in mice [11,86,129] only a study described the *ex vivo* use of aPDT to treat *S. aureus* skin infections was done [84]. Moreover, no studies were done to demonstrate the potential of using aPDT combined with antibiotics against *S. aureus* to increase the efficiency of bacterial inactivation.

In this study, an *ex vivo* porcine skin model was used to evaluate the aPDT efficacy against *S. aureus*. The results demonstrate that the inactivation of *S. aureus* by aPDT with a tetracationic porphyrin *in vitro* and *ex vivo* porcine skin model (1) was effective to inactivate *S. aureus*; (2) the efficacy of inactivation was concentration and light dose dependent; (3) the repetition of aPDT treatment increased significantly the bacterial inactivation; (4) the efficacy of *S. aureus* inactivation by aPDT in the presence of antibiotics increased significantly and that (5) this increase in aPDT efficacy was PS and antibiotic concentration dependent.

In vitro experiments a high bacterial concentration, around of 10^8 CFU mL^{-1}, which correspond to standard bacterial concentration used in other antimicrobial assays, such as antibiograms, was used and *S. aureus* was photoinactivated to the detection limit of the method (reduction of 8 log CFU) using a low PS concentration (5.0 μM) and a low irradiance (40 W m^{-2}). After 60 min of treatment, a reduction of 5 log was already observed. Similar results were obtained for this PS using other bacteria, namely a MRSA strain, under similar experimental conditions [49].

In *ex vivo* experiments, a lower concentration of bacteria was used, around 10^6 CFU mL^{-1}. As, in general, when bacteria are present in concentrations higher than 10^5 CFU mL^{-1} is considered a human infection [130,131], the *ex vivo* bacterial concentration tested in this study correspond to a skin infection situation. In this case, the maximal bacterial reduction was 4 log CFU, but a much higher PS concentration (50 μM) and irradiance (150 W m^{-2}) were necessary. The maximal of bacterial inactivation was higher than the 3 log CFU reduction (killing efficiency of 99.9% or more) established by the American Society of Microbiology to any new approach to be termed “antibacterial” [132]. However, under the same conditions, but using the PS at 25 μM, no significant bacterial inactivation was observed (reduction below 0.5 CFU log). Also, under low light dose, 40 W m^{-2}, no significant

bacterial inactivation was observed, even at 50 μM (reduction of around 1 CFU log). Overall, it was necessary to use a 10× fold higher concentrations and a 40x fold higher irradiance in this *ex vivo* model as compared to *in vitro*. Similar results were already observed by Maisch et al. (2007) [84]. These authors showed that a 100× fold higher concentration of a porphyrin derivative was sufficient to inactivate a MRSA strain in *ex vivo* porcine skin than *in vitro*. This is, however, expected because *in vitro* bacteria and PS are in solution, which allow that, at a given concentration, more PS molecules could bind to the surface of each bacterium resulting in a higher yield of ROS near the bacteria and therefore a higher antibacterial efficacy.

In the *ex vivo* skin model, bacteria are trapped in the tissue and, consequently PS molecules do not bind so efficiently to the bacterial cells. Besides, the PS can also adsorb to skin cells, being by this way less PS available to inactivate the bacteria. In fact, it is well know that the efficiency of aPDT is lower in samples with high contents of organic matter or cells. Lasocki et al. (1999) studied the antibacterial photodynamic effect of a hematoporphyrin derivative using *P. aeruginosa* and *S. aureus* either as suspension cultures in nutrient broth or as colonies growing on isolated mouse muscles [133]. The antibacterial effect was 1000 times greater in the case of suspension cultures and the concentration of PS necessary for optimal effects was much lower. Moreover, light penetration is more difficult in skin than that observed *in vitro*, in the buffer solution, reducing also the ROS production. These results highlight the importance of testing the efficacy of aPDT to inactivate bacteria in clinically relevant setting, such as porcine skin, which has been proposed as a good test model for human skin. Porcine skin has similar histological, physiological and immunological properties to human skin and has been suggested as a good analogue for medical research [134,135].

Although a higher PS concentration and a higher light dose were used in *ex vivo* skin model compared to *in vitro*, *S. aureus* after a cycle of treatment was not completely inactivated. After one aPDT cycle of 180 min with 50 μM of porphyrin under 150 W m^{-2} in porcine skin, around 2 CFU log of bacteria survive. However, when more two cycles of aPDT was applied, *S. aureus* was inactivated to the detection limit of the method, the remained 2 CFU log of bacteria after the first cycle were efficiently inactivated after two more cycles of treatment. Similar results have been observed in other *in vivo* studies. For instance Souza, et al. (2014) showed that to treat by aPDT toenail onychomycosis with

methylene blue, several sessions with an interval of 15 days between each session for 6 months, were done [136]. However, when antibiotics therapy is used to control bacterial infections and prevent relapse, several doses of antibiotics are administered. In general, antibiotic therapy has been conducted for 7 or 10 days with more than one dose by day. Nevertheless, it has been stated that longer exposure to antibiotics can contribute to resistance, causing also risks and harms for the patient [137–139]. But, if a person takes an inadequate course of antibiotics, they may relapse and require further treatment [140,141]. This increases the risk of developing resistance, as it would expose the person to antibiotics for longer. Conversely, the resistance problem does not arise for aPDT due to its mode of action and type of biochemical targets (multi-target process). So, the use of more than one treatment cycle to treat an infection could be a strong option. Moreover, in this study, it was only tested a series of 3 cycles of treatment and no viable bacteria were detected. Maybe 2 cycles could be enough to control the bacterial growth on porcine skin model.

Although the successful use of antibiotics is compromised by the potential development of resistance, their use is yet the first treatment option to treat and sometimes to eradicate diseases caused pathogenic bacteria such as *S. aureus* [28,78]. Now many of *S. aureus* strains are resistant to beta-lactams, macrolides, and even vancomycin, the ‘‘drug of last resort’’[70,79]. However, although it is well known that the use of these antibiotics give rise to the selection of antibiotic-resistant strains, avoiding bacterial inactivation, little effort has been made to employ aPDT in order to increase the efficacy of such antibiotics as well as to employ antibiotics to potentiate the aPDT efficacy.

In this work aPDT was tested *in vitro* and *ex vivo* in the presence of five antibiotics (ampicillin, chloramphenicol, penicillin, kanamycin and tetracycline) used to treat bacterial infections by *S. aureus* [99,127]. From these, ampicillin and penicillin interfere with bacterial cell wall synthesis and the other three inhibit bacterial protein synthesis. Chloramphenicol and tetracycline are bacteriostatic and the other three are bactericidal. The results showed that at MIC concentration none of these antibiotics enhance the efficacy of aPDT. The efficiency of photoinactivation with and without antibiotics was similar. However, when the five antibiotics were tested alone, the bacterial concentration was not affected after 180 min, even when the bactericidal antibiotics were used. Nevertheless, when the susceptibility of bacteria to these antibiotics was tested using the *susceptibility test discs* method, the five disks showed large zones of inhibition, indicating that the *S. aureus* strain

is susceptible to the tested antibiotic. As the *in vitro* experiments were done in a buffer solution, a long incubation period would be necessary to observe decrease of bacterial concentration. Having into account these results, one of the antibiotics, the ampicillin, was tested at higher concentrations, corresponding to 2x and 4x the MIC in vitro and 4x and 20x the MIC *ex vivo*. The choice took into account the fact that this antibiotic is bactericidal and interferes with bacterial cell wall synthesis, which is a target of aPDT. Being the main target of aPDT the external structures of the bacteria [122], cell membrane and cell wall, are destabilized allowing an easier enter of the antibiotics into the bacterial cells. In fact, the efficacy of *S. aureus* inactivation increased when aPDT was done in the presence of ampicillin *in vitro* and *ex vivo*.

In vitro, the synergistic effect was detected for the two antibiotic concentrations, but the photoinactivation in the presence of the higher concentration of ampicillin was observed sooner (reduction of 8 CFU log after 30 min of treatment) than with the lower concentration (reduction of 8 CFU log after 60 min of inactivation). When aPDT was done without the antibiotic, a similar bacterial reduction of 8 CFU log was obtained but only after 90 min of treatment. When the antibiotic was tested alone, no bacterial reduction was observed, even at the highest concentration, 1.0 μg mL^{-1}. Similar results were observed using the same PS and antibiotic for MDR *S. aureus*, *Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [82] and using other PS using vancomycin for vancomycin-sensitive and vancomycin-resistant enterococci [124] and methicillin, ampicillin, polymyxin B nonapeptide and tetracycline for both Gram-positive multi-resistant bacteria (*S. aureus*) and Gram-negative multi-resistant bacteria (*E. coli*) and for the disruption of *S. aureus* biofilms [27]. It is important to note that the synergistic effect of both therapies was only observed when aPDT alone cause a significant reduction in the bacterial concentration. For both antibiotic concentrations after 15 min of treatment, when no aPDT inactivation was still observed, no synergistic effect was detected, but after 30 min, when aPDT inactivation was already noticed, the synergistic effect was also observed.

In *ex vivo*, a similar pattern of variation was observed but only for the highest concentrations (5 μg mL^{-1}) of antibiotic. aPDT with antibiotic at 5 μg mL^{-1}, bacterial concentration was reduced to the detection limit of the method (a reduction of 6 CFU log after 180 min). The reduction with aPDT alone in the same conditions was 4 CFU log after 180 min of treatment. When the antibiotic was used alone, the maximal inactivation was of

1 CFU log after 180 min. At 1.0 μg mL^{-1}, no synergistic effect was observed. As for the *in vitro* experiments, the synergistic effect of both therapies was only observed when aPDT inactivation was observed, that is, after 90 min of treatment. This indicate that, as the main target of aPDT are the external structures of the bacteria [122], cell membrane and cell wall, these structures are destabilized during aPDT allowing an easier enter of the antibiotics into the cells block internal processes (inhibiting for instance protein synthesis that can be the antibiotic target, like chloramphenicol) and, naturally, improving the action of antibiotics which have the external structures as its target (like ampicillin). By this way, a synergistic effect between aPDT and antibiotics can be observed.

For low antibiotic and PS concentrations no synergistic effect was observed in the *S. aureus* inactivation. *In vitro*, an antibiotic concentration of 1.0 μg mL^{-1} (corresponding to 2x MIC) was necessary increase the efficacy of aPDT at 5.0 μM of PS. In *ex vivo*, a concentration of 5 μg mL^{-1} of ampicillin (corresponding to 20x MIC) and of 50 μM of PS was required to observe a synergistic effect in bacterial inactivation. Reducing the concentration of PS to 40 μM and maintaining the concentration of the antibiotic, no significant effect was observed relatively to aPDT without antibiotic. In the experiments with PS at 50 μM and PS 40 μM, the profile of aPDT inactivation without antibiotic was different in the first exposure times (60 min to 120 min), but at the end of the treatment the inactivation was similar. This different profile of inactivation was due to the use of different providers of porcine skin (in the experiments of irradiation at 150 W m^{-2} with a concentration 50 μM of Tetra-Py^{+}-Me and ampicillin in concentrations of 5 μg mL^{-1} a different provider of skin was used in the other experiments a same provider was used).

The results suggest that both therapies can increase the efficacy of bacterial inactivation, allowing the reduction of porcine skin treatment time for a quarter, but the reduction in PS or antibiotic concentration avoid the synergistic effect of the two therapies.

One aspect to be considered when aPDT is used to inactivate microorganisms in clinic is the selectivity of the PS for the microbes, avoiding an unacceptable degree of host tissue damage in the area of infection. With the exception of the prodrug 5-aminolevulinic acid (5-ALA), the precursor of protoporphyrin IX (PpIX), *in situ* and *in vivo* aPDT assays show that, in general, negative effects of PS on host cells do not occur when the PS is used at concentrations that are effective to microbial inactivation [142]. Furthermore, studies with the 5-ALA has shown that skin toxicity is the only relevant adverse effect known so far.

During light exposures, patients may experience pain in the irradiated region. Likewise, the time of incubation necessary for uptake and metabolism of the precursor and the long-lasting skin photosensitivity could be also other adverse effects of aPDT by 5-ALA [143]. In addition, the other antibacterial approaches, such as antibiotics application has also negative effects to the host. Nevertheless, porphyrins, such as the PS tested in this study, do not cause adverse effects on host cells [43]. Photodynamic inhibition of microorganisms occurs at porphyrin concentrations lower than those found to be cytotoxic for the host mammalian cells [43,144]. In fact, in this study, at the naked eye, no negative effects on the porcine skin were observed after 180 min of aPDT treatment with 50 μM of porphyrin under 150 W m^{-2}, suggesting that the porcine skin is still vital. However, more physiological studies are needed in order to evaluate the safety of the aPDT developed protocol. It is also increasingly necessary to assess the safety of aPDT directly on skin *in vivo*.

5. Future perspectives

After this work, it would be interesting to evaluate:

- the effect of other PS on *S. aureus* inactivation, especially PS mixtures with different ROS production pathways, for example the conjugation of tetracationic porphyrin, which produce mainly singlet oxygen to methylene blue that, contrary to the porphyrin, produces essentially free radicals;
- test the use of others antibiotics in sinergism with aPDT;
- the efficacy of two successive aPDT cycles to inactivate *S. aureus*;
- the efficacy of aPDT to inactivate methicillin-resistant S. *aureus* (MRSA) in skin model;
- use of different light condition, like red light (625 a 740 nm), once it penetrates deeper into tissues;
- the efficacy of aPDT to inactivate *S. aureus* "*in vivo"* (using an animal model the mouse).

6. References

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