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**Antimicrobial effect of different coupling of wavelengths and
dyes in photodynamic therapy protocols**

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Abstract

In recent years there has been a rapid increase in infections caused by antibiotic-resistant strains. Despite therapy, infectious diseases remain a leading cause of mortality and the growing phenomenon of drug resistance is an emerging problem.

Photodynamic Therapy (PDT) has been studied for antimicrobial purposes and antitumor applications.

In order to test PDT on fungal infections, we applied the same protocols *in vitro* on *Candida albicans* in planktonic cultures and in biofilms models, using also new molecules for antifungal therapy, and *in vivo* in a model of *C. albicans* infection in larvae of *Galleria mellonella*.

We performed PDT with 3 laser prototypes using 405, 532 and 650 nm wavelength with 3 fluences for *in vitro* planktonic cultures (10, 20 and 30 J/cm²) and one fluence of 10 J/cm² for *in vivo* studies on *G. mellonella* larvae and *in vitro* biofilms studies.

With regard to *C. albicans* cells suspensions, red diode laser used with toluidine blue caused a growth inhibition variable between 79.31% and 95.79%. The maximum inhibition of growth (100%) was obtained with the blue diode, at any used fluence, and curcumin. Green diode laser used with erythrosine caused a growth inhibition variable between 9.20% and 39.85%.

Larvae of *G. mellonella* infected with *C. albicans* SC5314 for every performed treatment showed a significant increase in survival in comparison to infected

animals inoculated with saline ($p<0.001$).

The combination of toluidine blue and red diode laser application led to a prolonged survival compared to dye alone or laser application alone, although the difference in survival was not statistically significant between the 3 groups. A statistically significant difference in survival was found between the group inoculated with curcumin alone compared to the group treated with blue diode laser coupled with curcumin ($p=0.02$) and between the group of larvae irradiated with green diode laser compared to the group treated with laser coupled with erythrosine ($p=0.03$).

Treatment performed with red diode laser and toluidine blue did not show any effect on *C. albicans* biofilm, as was for red diode laser alone and toluidine blue alone. Conversely, good results were found for green diode laser and erythrosine, with the maximal effect obtained with the combination of laser and dye ($p=0.0068$) and a significant result also for laser alone ($p=0.0131$). The association of blue diode laser and curcumin gave the best results in comparison with untreated control ($p<0.0001$), while curcumin alone showed better results than laser alone ($p=0.0057$).

In the comparison with the untreated control, the application on *C. albicans* biofilm of red diode laser with or without toluidine in combination with KP treatment showed a statistically significant result ($p<0.0001$), but the combination of dye and KP defined the same significant result without laser application ($p<0.0001$).

For the same comparison, the application of blue diode laser with curcumin in combination with KP treatment showed a statistically significant result ($p=0.0006$), but the combination of dye and KP defined the same significant result without laser application ($p=0.0001$).

Blue diode laser and curcumin were more efficient with KP than without KP ($p<0.0001$ vs untreated control) and blue diode laser and KP were more efficient with curcumin than without curcumin ($p=0.0065$ vs untreated control).

The application of green diode laser without erythrosine in combination with KP treatment showed a statistically significant result ($p=0.0002$) compared to the untreated control.

APDT may be a good alternative to antimicrobial drugs, given the possible acquired resistance, especially for the treatment of localized infections of the skin and oral cavity.

Abbreviations

AIDS: Acquired Immune Deficiency Syndrome
APDT: Antimicrobial PhotoDynamic Therapy
ATP: Adenosine TriPhosphate
CC: control non-activated essential oil
CE: natural and tungsten lights
Ce6: Chlorine e6 encapsulated in cationic CTAB-liposomes
CFU: Colony Forming Unit
CUR: Curcumin
CW: Continuous Wave
DMSO: Dimethyl Sulfoxide
E: indocyanine green alone
EO: *Citrus aurantifolia* essential oil
EOS: Eosin
ERY: Erythrosine
ICG: indocyanine green
IRL: Laser alone
IRLE: Indocyanine green (Emundo, 1 mg/ml)
KP: Synthetic decapeptide or Killer decapeptide
KTs: Killer Toxins
LASER: Light Amplification by Stimulated Emission of Radiation
LED: Light Emitting Diode
LLLT: Low Level Laser Therapy
MASER: Microwave Amplification by Stimulated Emission of Radiation
MB: Methylene Blue
MG: Malachite Green
MOPS: 3-(*N*-morpholino)propanesulfonic acid
NMB: EmunDo® or new methylene blue
OD: Optical Density
PaKT: *Pichia anomala* Killer Toxins
PBS: Phosphate Buffered Saline
PDT: PhotoDynamic Therapy
PMMA: PolyMethyl Methacrylate
PS: Photosensitizer
RB: Rose Bengal
ROS: Reactive Oxygen Species
RPMI: Roswell Park Memorial Institute
SD: Standard Deviation

SDA: Sabouraud Dextrose Agar

SEM: Scanning Electron Microscopy

TB: Toluidine blue

TMP-1363: meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate

XTT: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide

YAG: Yttrium Aluminium Garnet

ZNPC: cationic nanoemulsion of zinc 2,9,16,23 tetrakis(phenylthio)-29*H*,31*H*-phthalocyanine

Introduction

In recent years a rapid increase in infections caused by antibiotic-resistant strains was observed. Despite therapy, infectious diseases remain a leading cause of mortality: bacterial infections cause 17 million deaths globally (Butler MS et al 2006).

Candida albicans is the most prevalent fungal pathogen in humans and, because of the growing phenomenon of drug resistance, the need for new antifungal agents for the management of *C. albicans* infections is an emerging problem (Zida A et al 2016).

Diverse microorganisms, Gram-positive, Gram-negative, aerobic and anaerobic bacteria, mycoplasmas, fungi and protozoa colonize the oral cavity. Dental plaque can be defined as a community of various microorganisms present on the surface of the tooth as biofilms, embedded in an extracellular matrix of polymers of microbial origin. Scientific literature describes an increased resistance to antimicrobial drugs by microbial biofilms (Sardi JCO et al 2013).

The limited access of the plaque to topical agents and the development of antibiotic and antimycotic resistance create the need for alternative control strategies to treat infectious diseases also in the oral cavity.

In dentistry, the Antimicrobial Photodynamic Therapy (APDT) is used with different types of applications, different types of laser and different photosensitizers (Rolim JP et al 2012).

The cytotoxic effect is achieved through the local application or systemic administration (oral or intravenous) of photosensitizing agents followed by irradiation of visible light with emission spectrum appropriate to the absorption spectrum of the used photosensitizer, in the presence of oxygen (Knopka K et al 2007). This induces oxidation phenomena with irreversible consequence of selective destruction of proteins, lipids, nucleic acids and other cellular components (Valenzano M et al 2007).

There are numerous references for treatments with He-Ne laser, diode laser for the treatment of periodontitis, peri-implantitis, endodontics, also in association with the technique of Guided Bone Regeneration (Williams JA et al 2006, Haas R et al 2000).

Light Amplification by Stimulated Emission of Radiation: LASER

Since 1917 Albert Einstein with the “stimulated emission theory”, which is the basis of the amplification processes and molecular oscillation and thus of the interaction between light and matter, built the basis for the creation of a revolutionary technology, the LASER, name representing the acronym for Light Amplification by Stimulated Emission of Radiation (Fornaini C and Rocca JP 2015). The creation of the laser was preceded by few years (1954) from that of another instrument, the MASER (Microwave Amplification by Stimulated Emission of Radiation), a sort of precursor of laser having a practical application in the improvement of communication systems, in particular in the navigation.

Townes and Schawlow were awarded for this invention of the Nobel Prize in Physics in 1981.

Theodor Maiman in 1960 created the first laser device, a pulsed ruby laser. Since then the technological evolution of these devices took place very quickly so that in our time the laser is applied in many fields, from medicine to industry, to the military communications.

In addition to the so-called “spontaneous emission”, Einstein described an emission called “stimulated” characterized by the production of two identical photons instead of the single photon produced with the spontaneous emission (Figure 1). To produce a real amplification of the light, the number of atoms in excited state must be greater than the number of atoms in the fundamental state: only in the moment during which this “population inversion” is produced through a pumping system, the stimulated emission can occur with an amplification phenomenon. To produce a laser emission, the presence of at least three energy levels is necessary (Ground State or GS, N1 and N2). This is due to the pumping system that creates an excitation in the transition from the ground state GS to the energy level N2 since the decay N1 to the ground state is particularly rapid; the N1-N2 passage is instead a metastable state of long duration.

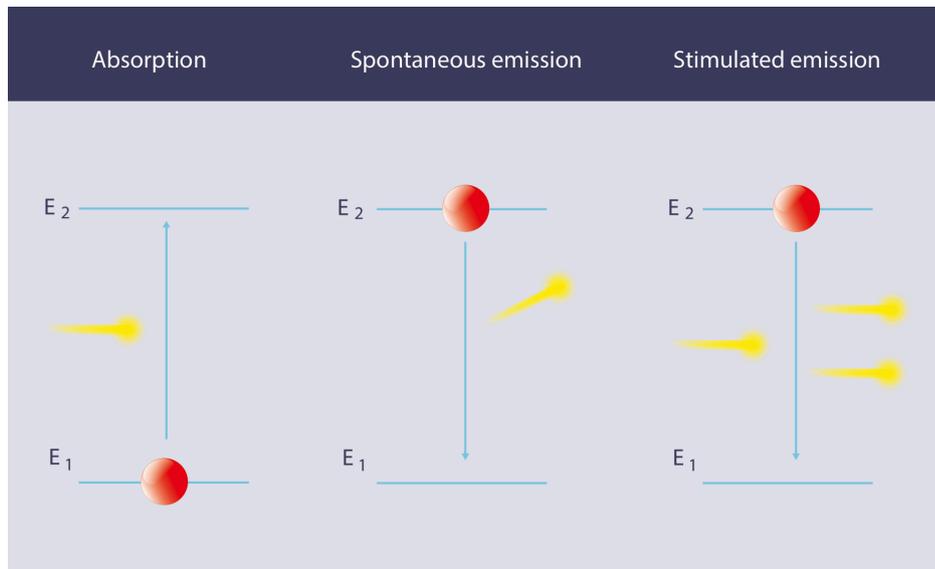


Figure 1. Absorption, spontaneous and stimulated emission (Fornaini C and Rocca JP 2015).

A laser source consists of three basic elements, namely an active medium, an optical cavity and a pumping system.

The active medium consists of a series of atoms or molecules that, excited, give rise to the phenomenon of population inversion, and, subsequently, to a stimulated emission. The active medium may be solid, liquid or gaseous, and is the decisive element for the wavelength of a laser: in dentistry, lasers are mainly constituted by an Yttrium Aluminum Garnet (YAG) crystal, in which a small portion of the molecules is replaced by elements of the group of rare earth element such as erbium (Er) or neodymium (Nd) from which the conventional names Er:YAG and Nd:YAG.

An interesting example of active gaseous medium is the CO₂ laser introduced in dentistry by Frame in 1980.

A further group of laser is that of diodes or semiconductor lasers, particularly advantageous for small size, low costs and easy maintenance.

Pumping system is the laser part providing energy to the atoms or molecules of the active medium to cause the population inversion or the maintenance of a greater number of atoms in excited state compared to the so-called “ground state” or baseline state.

It can be of three types:

- a) Optical pumping may be a xenon lamp, a flash lamp or another laser.
- b) Electric pumping is consisting of an electric discharge and it is used with lasers in which the active medium is a gas.
- c) Chemical pumping is the system using a chemical reaction.

Optical cavity or Fabry-Perot cavity is formed by two parallel mirrors, one reflective at 100%, the other one at 95%, within which the active medium is located.

Light bounces back and forth between the mirrors, gaining intensity with each pass through the medium and finally comes out from the 95% reflective mirror creating the laser emission.

PROPERTIES OF LASER LIGHT

The laser light has unique characteristics that distinguish it from ordinary light, such as sunlight or that of an incandescent lamp. Those characteristics are: monochromaticity, coherence and collimation (Figure 2).

MONOCHROMATICITY

Unlike the ordinary light, which can be decomposed into a spectrum of colors, the laser has only one wavelength, then only one vibration frequency, then only

one color, characteristic of the active medium which has produced it; this characteristic explains why, sometimes, lasers are identified by means of their wavelength instead of the active medium (for example, Er:YAG or 2,940 nm).

COHERENCE

All photons vibrate in phase, in space and in time, according to the stimulated emission theory of Einstein, unlike the ordinary light where photons move randomly without coherence of phase or direction.

COLLIMATION

The radiation exits from the laser in a certain direction and spreads with an extremely small angle of divergence (in the order of milli-radians).

BRIGHTNESS

Also said radiance, brightness is the power emitted by the unit surface under a solid angle of observation and is measured in $\text{W}/\text{m}^2/\text{steradian}$. At the moment, with no other equipment it is possible to obtain such high field strengths: consider, for example, that a He-Ne laser has a brightness equal to 300 times that of the sun.

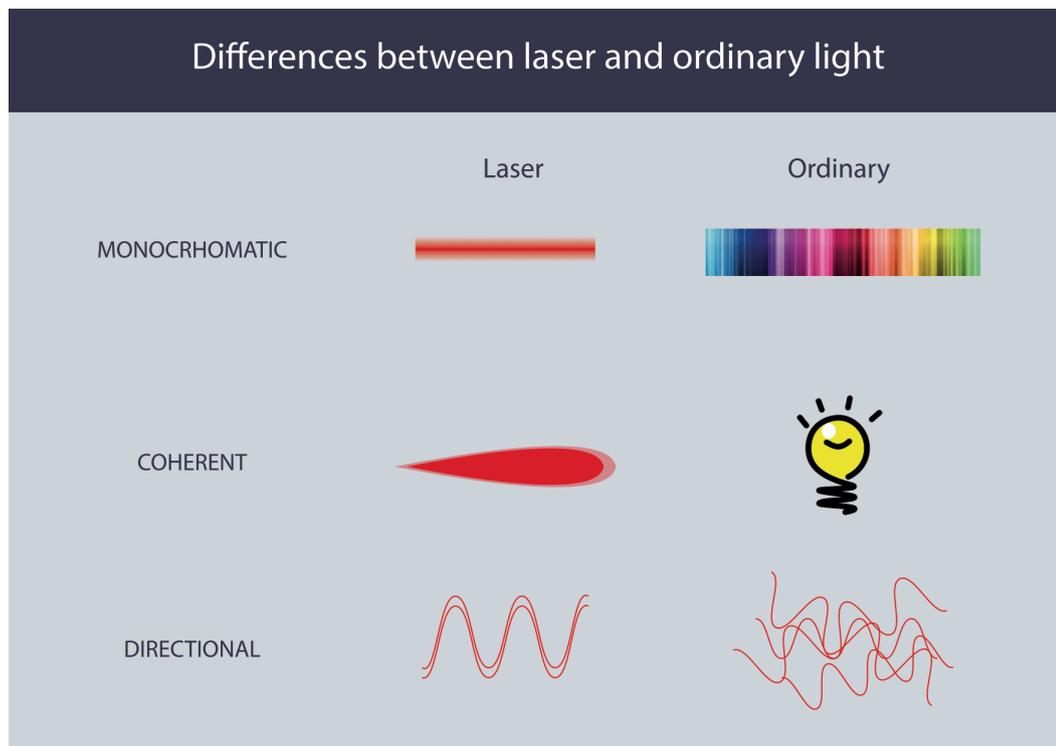


Figure 2. Characteristics of ordinary and laser light (Fornaini C and Rocca JP 2015).

PARAMETERS OF THE LASER BEAM

Wavelength: it depends on the active medium and represents the spatial periodicity. It is expressed in nanometers. The wavelengths used in dentistry range from 400 nm (visible) in the blue-purple approximately to 10,600 nm, the laser (infrared). Moreover are used wavelengths ranging from visible light (400 nm – 780 nm) and infrared light (greater than 780 nm).

Frequency: for laser working in pulsed mode, frequency represents the number of pulses per second and is expressed in Hertz.

Power: expressed in Watts. The peak power is the maximum power obtainable with one pulse. The average power (pulsed lasers) represents the average

between the periods during which the laser emits and stops emitting (between two pulses). The power density (PD) is expressed as follows: $PD=W/cm^2$.

Energy: it is expressed in Joules ($J = W \times sec$). Fluence (F) or energy density is the energy delivered per surface expressed in cm^2 . So $F=J/cm^2$. This is a particularly important parameter because it is able to fully describe a laser treatment and to compare different devices (different pulse durations, different handpieces, ...).

TRANSMISSION SYSTEMS

They are systems for the transport of the laser light from the optical cavity to the target, e.g. the oral cavity. The ideal characteristics of the lowest loss of energy associated to the greatest flexibility of the system.

Fixed lens: a series of lenses fixed on a rigid support in a highly efficient but lowly flexible system.

Articulated arms: this system, characterized by a series of tubes connected by a series of mirrors, has a better flexibility than the fixed lenses, but an efficiency of about 90%.

Hollow fibers: these fibers have an internal reflective surface which makes the system particularly free during movements; however, they are characterized by two disadvantages: the loss of energy bending the tube and the limited duration.

Optical fibers: they are made of two parts, an outer one (cladding) and an inner one (core) with different refraction indexes; they are frequently made of quartz, have high flexibility and a diameter variable from 200 to 900 micrometers. The

main disadvantage is the relatively low efficiency, especially for wavelengths absorbed in water (example: Er:YAG laser).

Direct delivery system: is the latest laser transmission system created to limit the energy loss due to the transfer of the laser light from optical cavity all along the fiber or the articulated arm. In this technology laser power is generated at the lower part of the handpiece where the active medium (e.g. Er: YAG bar) is located with the pumping system (e.g. flashlamp), allowing for a greater energy transfer.

EMISSION MODE

Lasers can transmit energy in two different modes: in continuous mode or continuous wave (CW) or in pulsed mode (Figure 3).

In the case of continuous mode the beam is emitted without interruption, in a continuous manner precisely, maintaining the power at a constant level so that the peak power and average power coincide.

The pulsed mode: the laser light emission is made of periods of interruption so that the peak power is always greater than the average power and the increase of the temperature in the target tissue is controllable (thermal relaxation). The relationship between the working period and the pause period is called duty cycle.

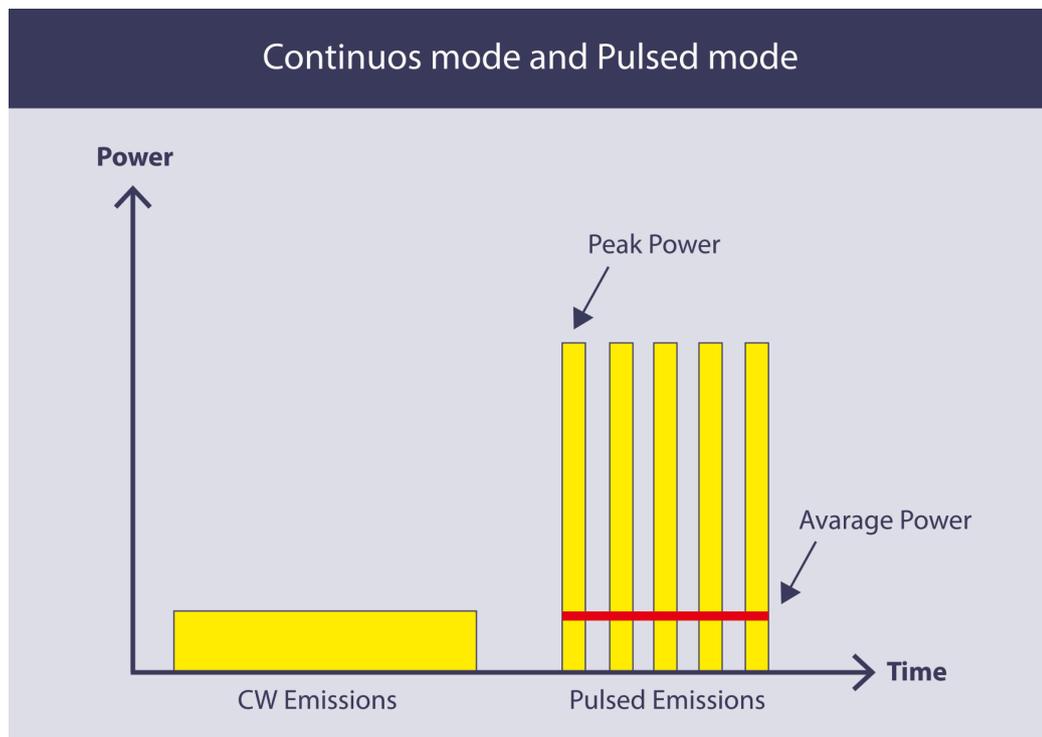


Figure 3. Continuous and pulsed mode in laser emission (Fornaini C and Rocca JP 2015).

While Er:YAG or Nd:YAG lasers can emit only in the pulsed mode, other lasers, such as the CO₂ laser, can emit in both modes on the basis of the operator choice.

Mistakenly, the term “pulsed mode” is often used for diode laser: in this case the output mode, more properly defined “chopped” or interrupted, is linked to the interruption of a continuous mode by means of devices as disks or choppers.

There are, then, two further emission modes, the Q-switched mode that uses a device (rotating mirror, switches, ...) capable of opening up and giving a large number of photons that were accumulated in the Fabry-Perot cavity.

LASER-TISSUE INTERACTIONS

The laser light when interacting with an object or a tissue undergoes four possible interactions (Figure 4), namely:

- Transmission: light passes through the matter without interacting with it.
- Absorption: the light is captured by the material in a way correlated to the wavelength and the absorption coefficient of the tissue; it is the most important interaction for therapeutic or diagnostic procedures and depends mainly on tissue chromophores, such as water, melanin and hemoglobin. The laser energy is transformed into heat by defining different degrees of thermal damage: hyperthermia (42-45°C), reduction of the enzymatic activity and protein denaturation (50-60 C), dehydration (100°C), vaporization and carbonization (>100 C), thermal ablation, and photoablation (300-1,000°C).

The majority of organic molecules has a high level of absorption for ultraviolet rays. Melanin and hemoglobin have a high absorption index of blue, green and yellow radiation. Red and near infrared have a deep penetration into the tissues.

Water and hydroxyapatite have a higher absorption for infrared rays.

- Diffusion or scattering: the beam propagates in all directions and is inversely proportional to the fourth power of the wavelength. The diffusion of photons is characterized by a change of direction of propagation in the areas adjacent to the laser-irradiated area without loss of energy. The scattering is the way in which light interacts with the material, in which the direction of the incident ray is

changed by the particles where it passes through. The diffusion plays an important role in the spatial distribution of the absorbed energy.

- Reflection: the beam reaches the surface and is reflected from it in a way linked to the angle of incidence. A precise knowledge of the reflectivity of the materials or tissues is very important especially when this has a high value. The reflection depends on the chemical composition of the medium: in the metal surfaces this index is very high. The reflected laser beams can cause damage to the skin and especially to the eyes: this is the reason why the use of safety glasses with specific lenses is required for patients, operators and staff.

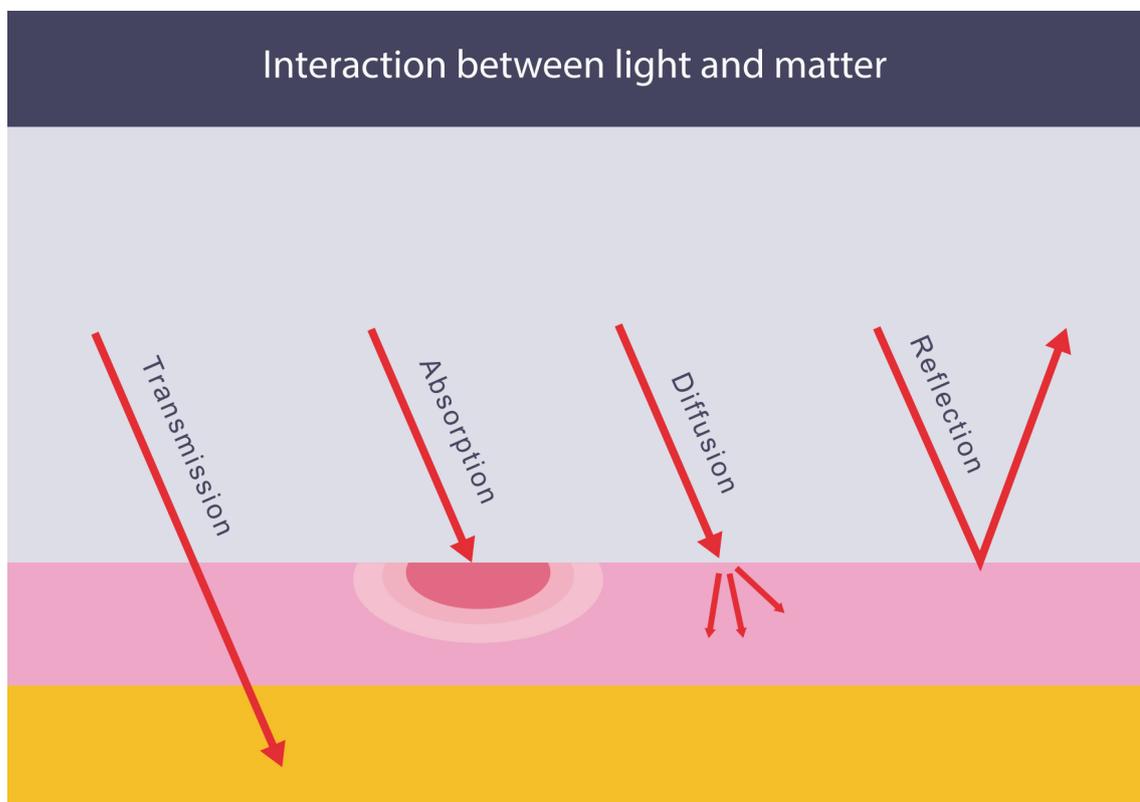


Figure 4. Light-matter interactions (Fornaini C and Rocca JP 2015).

The interaction of the different wavelengths with the tissues varies depending on their nature (mucosa, bone, enamel, dentin), their degree of hydration and vascularization, and the affinity or absorption coefficient.

Laser-tissue interactions can arise four different effects, namely photochemical, photothermal, photoablative and photomechanical effects, of which photochemical and photothermal are the two most important effects for medical applications (Figure 5).

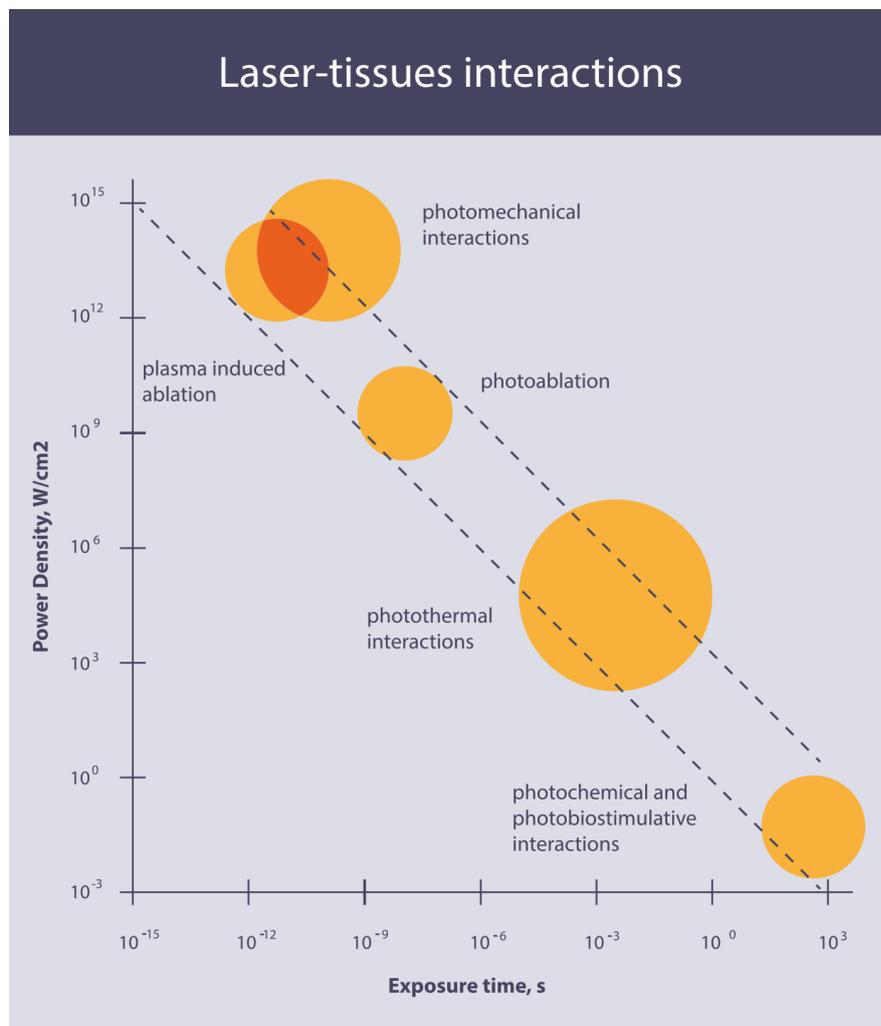


Figure 5. Laser-tissue interactions (Fornaini C and Rocca JP 2015).

- Photochemical effects: are due to activation of biochemical reactions and take place when the energy of the photons is greater than the energy of the chemical bonds. These effects allow clinical applications such as Low Level Laser Therapy (LLLT) or biomodulation in which the use of low power is able to produce bio-stimulation, analgesia and anti-inflammatory effects and muscle relaxants or photodynamic therapy (PDT) for antimicrobial or antitumoral applications.

- Photothermal effects: common to all wavelengths, are based on the conversion of optical radiation into thermal energy.

Effects are usually reached with power densities ranging around 100 W/cm^2 obtained by irradiation with pulsed lasers at pulse duration of microseconds or with laser working in continuous mode.

The photothermal effects allow to incise, excise, vaporize and coagulate in a mode depending on the type of laser and the affinity of the wavelength to the target tissue.

Lasers in the spectrum of visible and near infrared, such as argon laser, KTP, diode, and Nd:YAG, are well absorbed by chromophores such as hemoglobin and melanin. The lasers belonging to the portion of mid and far infrared as Er:YAG and CO_2 , have, however, clear affinity for water and hydroxyapatite.

The first are mainly used on soft tissues (incision, vaporization and coagulation), while the latter are used both on hard tissue (ablation) and soft tissues (incision

and vaporization of the water content), but with less hemostatic effect because of the lack of affinity with the hemoglobin.

Photodynamic therapy

History

Phototherapy began in ancient Greece, Egypt, and India, but disappeared for many centuries and had rediscovered by the Western civilization at the beginning of the 20th century when a Danish physician, Niels Finsen, demonstrated the efficacy of an arc lamp, the so-called “Finsen lamp” for the treatment of tuberculosis and Lupus Vulgaris. Thanks to this discovery, Finsen won the Nobel Prize in 1903.

Quite at the same time, over 100 years ago, Oskar Raab, a medical student working with Professor Herman Von Tappeiner in Munich, discovered PDT by chance (Mitton D et al 2008, Dolmans DF et al 2003) through the observation of the lethal effects of the combination of acridine red and light *Paramecium* cultures (Deniell MD and Hill JS 1991; Raab O 1900).

PDT has also been studied in order to obtain the destruction of tumors, through the death of tumor cells by necrosis or apoptosis, the damage of the microcirculation of the tumor and the activation of an immune response against tumor cells (Dolmans DE et al 2003). Thomas Dougherty and co-workers at Roswell Park Cancer Institute of Buffalo, New York, tested PDT clinically, and published in 1978 their results on a large number of cutaneous or subcutaneous

malignant tumors with a total or partial resolution of most of them (Rajesh S et al 2011).

Mechanism

In order to obtain a photodynamic reaction three basic elements must be involved (Issa MCA et al 2010):

1. A photosensitizer (PS) i.e. a photosensitive molecule localized in a cell or in a target tissue.
2. A light source with specific wavelength, required to activate the photosensitizing molecule.
3. Molecular oxygen, which is essential for Reactive Oxygen Species (ROS) generation.

Many organic molecules of biological origin can act as photosensitizers as they present a good quantum yield of triplet formation and a lifetime of this excited state relatively long (even hundreds of microseconds).

The requirements of an optimal photosensitizer are multiple: it should be non-toxic and show local toxicity only after activation with light, it should have highly selective accumulation and high quantum yield of singlet oxygen production (Allison RR et al 2004a, Meisel P and Kocher T 2005).

Among the first molecules used as photosensitizing agents in living organisms with visible light, there were some agents of natural origin such as porphyrins (Issa MCA et al 2010).

The porphyrins, like all chromophores, undergo electronic excitation, following the absorption of a quantum of light, passing by the fundamental electronic state to a higher level of electron energy, the excited singlet (Juzeniene A et al 2004).

The absorption spectrum of these molecules varies from 400 to 700 nm; for this reason porphyrins are optimal photosensitizers for applications in this field (Reddi E et al 1988).

PDT requires a light source that activates the photosensitizer by exposure to low-power visible light at a specific wavelength. The most part of photosensitizers is activated by red light between 630 and 700 nm (Salva KA 2002) and the most used photosensitizers in APDT are methylene blue (MB), toluidine blue (TB), erythrosine (ERY), rose Bengal (RB), eosin (EOS) and malachite green (MG) (Rolim JP et al 2012, Vilela SF et al 2012, Junqueira JC et al 2010).

The antimicrobial activity of photosensitizers is mediated by singlet oxygen, which, because of its high chemical reactivity, has a direct effect on the extracellular molecules. In recent years, systems based on Light-Emitting Diode (LED) technology have been developed (Vilela SF et al 2012).

In the past, the activation of the photosensitizer was obtained by means of different light sources, such as argon laser, but it is more common to use visible diode lasers that are cheaper than the first one and more easy to handle and transport. Same remark may be expressed for LED lights (Kubler AC 2005,

Allison RR et al 2004 b, Juzeniene A et al 2004, Pieslinger A et al 2006, Steiner R 2006).

The mechanism of action involves the absorption of a photon of appropriated light leading to excitation of the photosensitizer to its short-lived excited singlet electronic state. This singlet-state PS can undergo an electronic transition to a much longer-lived (microseconds) triplet state. The longer lifetime allows the triplet PS to react with ambient (ground state) oxygen by one of two different photochemical pathways, called Type 1 and Type 2. Type 1 involves an electron transfer to produce superoxide radical and then hydroxyl radicals (HO°), while Type 2 involves energy transfer to produce excited state singlet oxygen ($^1\text{O}_2$). Both HO° and $^1\text{O}_2$ are highly ROS that can damage all types of biomolecules (proteins, lipids and nucleic acids) and kill cells (Figure 6) (Hamblin MR 2016, Dai T et al 2012).

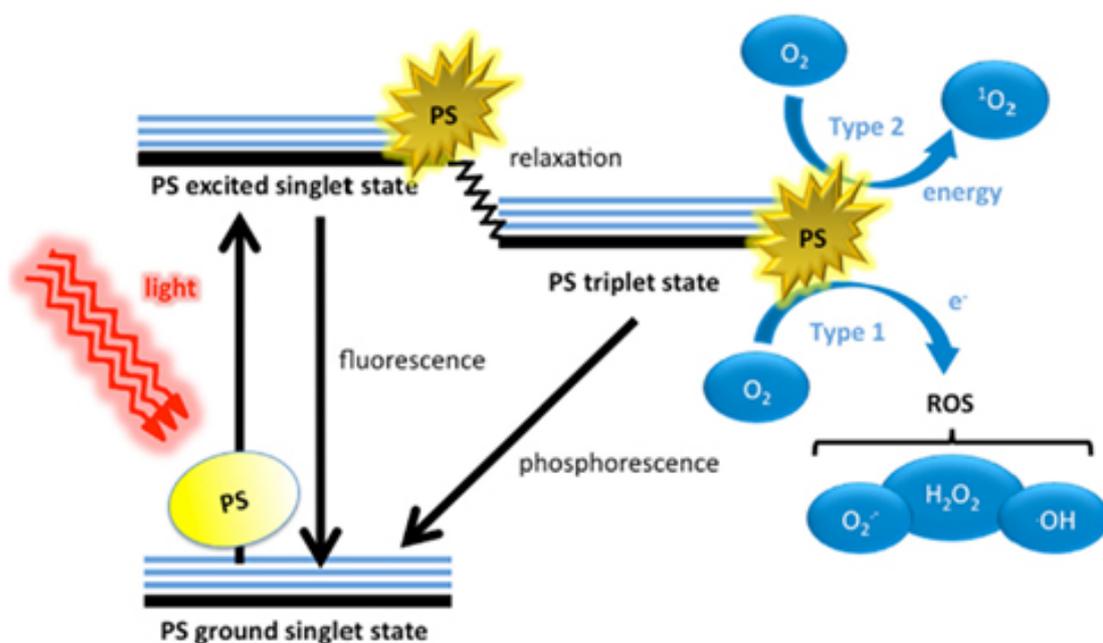


Figure 6. Mechanisms of PDT (Dai T et al 2012).

***Candida* and candidosis**

Fungal infections have an important impact on human health particularly because of the growing number of immunocompromised patients for AIDS, organ transplantation and cancer chemotherapies, and widespread antibiotic use (Sellam A and Whiteway M 2016). *Candida* species, particularly *C. albicans*, are the fourth most common cause of nosocomial infections in North American hospitals (Yapar N 2014) and the fourth most common cause of bloodstream infection (Nett JE et al 2016).

Recently, more advances have been made in the understanding of mechanisms by which *C. albicans* increases its virulence, such as biofilm formation, stress response, and metabolic adaptation (Manfredi M et al 2013; Sellam A and Whiteway M 2016).

Response to stress is a critical function for opportunistic pathogens because of the ability to overcome host defenses through ubiquitous heat shock proteins.

The transition of *Candida* spp. from harmless commensals to pathogenic microorganisms is most often related to a weakening of the host immune defences.

Candidosis are more frequently observed at superficial level, but in immunocompromised patients, candidosis can be systemic and highly associated to death: mortality due to invasive candidosis is estimated at 26-38% (Williams D and Lewis M 2011; Nett JE et al 2016).

Oral candidoses are classified in three different types such as acute and chronic pseudomembranous, erythematous and chronic hyperplastic candidosis and other manifestations so-called *Candida*-associated lesions as denture stomatitis, angular cheilitis, median rhomboid glossitis and linear gingival erythema (Williams D and Lewis M 2011).

***Candida* biofilms**

Biofilm is the predominant growth state of many microorganisms and is identifiable as a community of adherent cells with properties that are distinct from those of free-floating or planktonic cells, particularly for the greater resistance of the cells to chemical and physical insults (Nobile CJ and Johnson AD 2015).

C. albicans produces highly structured biofilms composed of multiple cell types (i.e., round, budding yeast-form cells; oval pseudohyphal cells; and elongated hyphal cells) encased in an extracellular matrix (Fox EP and Nobile CJ 2012, Nobile CJ et al 2012).

Biofilm formation and maintenance are related to different characteristics and properties as adherence, dimorphism and production of extracellular matrix. Adherence is the ability of cells to adhere each other and to surfaces, and it is linked to many transcriptional regulators (Nobile CJ and Johnson AD2015).

Dimorphism of *C. albicans*, i.e. the ability to form hyphae or yeast cells, is important to biofilm formation because the capability of hyphae to contribute to

the mechanical and architectural stability of biofilm and to support yeast cells (Nobile CJ and Johnson AD 2015).

Extracellular matrix, mainly composed of proteins and glycoproteins (55%), plays an important role in drug resistance because it acts as physical barrier to drug penetration (Nobile CJ and Johnson AD 2015).

In patients wearing dentures, biofilm constituted by bacteria and *Candida* cells can be formed both on the oral mucosa and on the surface of dentures, commonly leading to acute or chronic candidosis, including denture stomatitis. *C. albicans* biofilm formation is initiated when planktonic yeasts adhere to a surface and begin to aggregate and form microcolonies.

This first stage, which is vital for biofilm formation, is immediately followed by a proliferation of yeast cells and the beginning of hyphal development (Chevalier M et al 2012).

Aims of the Study

The first aim of this study was to test PDT protocols against planktonic *C. albicans* cultures suspended in saline solution or growing on solid medium.

In the second part of the study, the aim was to optimize the model of *G. mellonella* to test the application in PDT protocols of different laser wavelengths in combination or not with different photosensitizing dyes in *C. albicans* infection.

The main objective of the last part of the study was to apply potential therapeutic strategies alternative to antifungal drugs to complexes *in vitro* biofilms mimicking the *in vivo* infection. Since oral prosthetic surfaces can be easily colonized, biofilms created on resin materials normally used in prosthetic dentistry were tested.

In addition, a further objective was to match the photodynamic therapy to new molecules, such as a synthetic antibody-derived peptide endowed with antimicrobial activity.

Materials and methods

Systematic review

A preliminary topic of this work was to understand the evidence for PDT on *C. albicans* in *in vitro* studies based on the use of laser devices.

Eligible papers were characterized as *in vitro* experimental studies that evaluated the use of laser photodynamic therapy on *C. albicans* planktonic or biofilm

cultures. The electronic search of scientific papers was conducted in the PubMed/Medline database including English, Italian or French language studies. The following descriptors were used separately and in combination: photodynamic therapy, laser, *C. albicans*.

As inclusion criteria, the articles needed to have availability of access to full text. The selection of studies was initiated by the review of the titles of articles identified through the search strategies. Articles whose titles did not reflect the purpose of this review were excluded. All other items were preselected and had their abstracts analyzed. The papers whose abstracts matched the theme or did not provide sufficient data for a clear decision had their full text reviewed. Finally, after reading the full text, the studies that met the aim of this review were included.

Dyes concentration and laser sources

Erythrosine and toluidine blue were dissolved in distilled sterile water, and curcumin in dimethyl sulfoxide (DMSO), at 20 mM concentration.

In the preliminary experiment, toxicity of dyes (photosensitizers) and the right concentration to use was evaluated.

1. Erythrosine 100 μM showed a toxicity of about 14.86% with respect to the control in saline solution and of about 8.95% for 100 μM and 6.71% for 10 μM for solid medium.

2. Curcumin revealed no toxicity in any situation.

3. Toluidine blue showed a toxicity related to the concentration in saline solution of about 92.97% for 100 μM and 0.80% for 10 μM . Moreover toluidine blue showed a greater toxicity in saline solution.

On the basis of these results, we decided to use a concentration of 100 μM for curcumin and erythrosine and 10 μM for toluidine blue.

This study has been realized with three different wavelengths in the visible spectrum of light used with or without photosensitizing dye coupled with the wavelength on the basis of colour affinity: we irradiated with a red diode (650 nm) cultures stained with toluidine blue, with a blue-violet diode (405 nm) cultures stained with curcumin and with a green diode (532 nm) cultures stained with erythrosine (Figure 7).

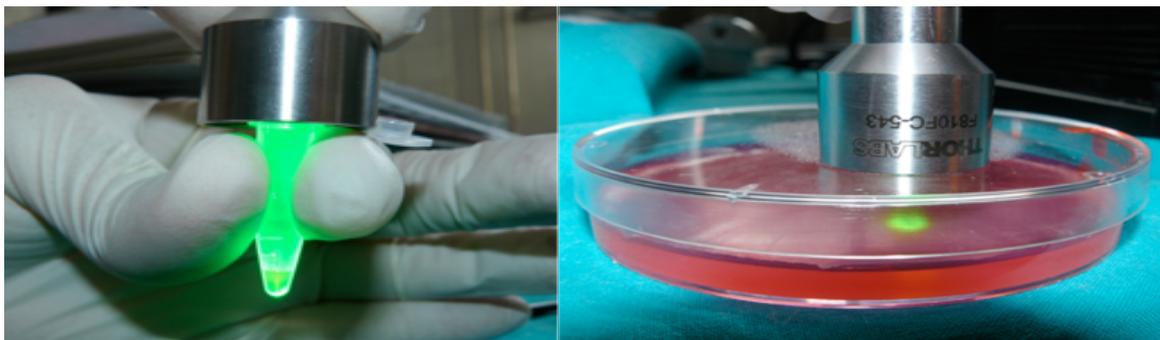


Figure 7. Example of laserization in liquid culture medium in Eppendorf tubes (left) and solid culture medium in agar plates (right).

For this study we used 3 laser prototypes that we tested with a power meter (PM-200, Thorlabs). On the basis of recorded power of each wavelength and of the fact that prototypes were not modifiable in terms of emitted power, we

planned the irradiation time for each condition, having chosen 3 different values of applied fluences: 10, 20 and 30 J/cm².

Laser irradiation has been performed in continuous mode for the different wavelengths.

We realized every laser application on *C. albicans* cells streaked on Sabouraud Dextrose agar (SDA) plates or suspended in saline solution (in Eppendorf tubes).

Agar plates were irradiated with the 3 parameters in a half of the plate, using the remaining one as a control (Figure 8).

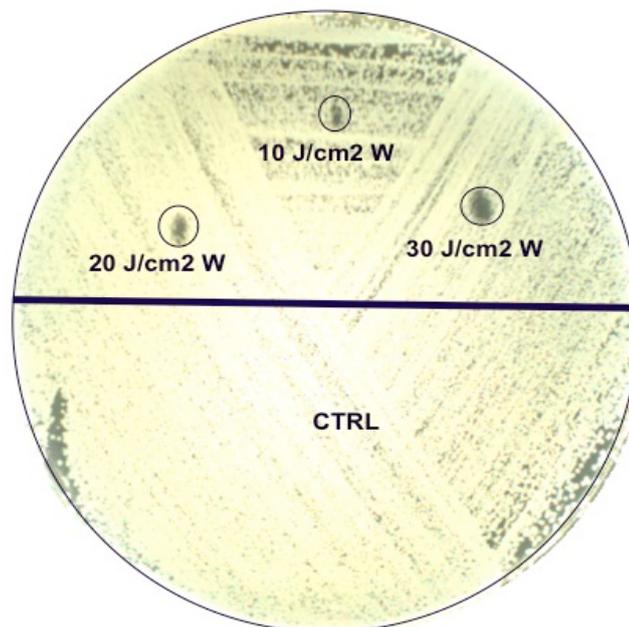


Figure 8. Irradiation mode of the plate.

In the case of liquid fungal suspensions (Eppendorf tubes), each parameter has been tested individually. All conditions have been realized in duplicate.

In summary then we have for each experimental condition 8 Eppendorf tubes, with and without dye; of these 8, 2 did not undergo to irradiation.

Laser 650 nm red diode was applied to 6 Eppendorf tubes with toluidine blue 10 μ M, 6 controls without dye and 2 plates with toluidine blue and 2 control plates. Out of the 6 Eppendorf tubes, 2 were treated with laser for 307 seconds, 2 for 615 seconds and 2 for 923 seconds. Likewise, each plate was treated for the 3 different times in different areas, on a half of the plate.

Laser blue diode 405 nm was applied to 6 Eppendorf tubes with curcumin 100 μ M: 2 for 50 seconds, 2 for 100 seconds and 2 for 150 seconds. It was then applied to the 6 controls without curcumin and 4 agar plates: 2 with curcumin and 2 without dye, while respecting the 3 different times in different areas of the same plate.

Laser green diode 532 nm was applied to 6 Eppendorf with erythrosine: 2 for 95 seconds, 2 for 190 seconds and 2 for 285 seconds. It was then applied to the 6 controls without erythrosine, 2 plates with erythrosine and 2 plates without erythrosine, respecting the 3 different times in different areas of the same plate.

Agar plates subjected to laser were incubated at 37°C in aerobic conditions and observed after 1 day for the presence of growth inhibition in the irradiated area.

Samples in Eppendorf tubes were prepared for counting: from every Eppendorf tube, 20 μ l were taken and streaked on a plate. With a sterile loop of polystyrene the liquid was spread over the entire surface and the plate was incubated at 37°C in aerobic conditions. After 1 day of incubation colonies were enumerated.

Non irradiated fungal suspensions were used as growth control. The antifungal effect was evaluated as percentual reduction of colony number in comparison to the control. The count of CFUs was carried out on the same day.

***In vivo* assay**

In this study larvae of *G. mellonella* have been used as non mammalian host for infection with *C. albicans* and treatment with lasers and dyes.

Larvae of *G. mellonella* have recently been used as model hosts for studying pathogenic microorganisms as an alternative to vertebrates (Chibebe JJ et al 2013). In 2010, Fuchs and colleagues reported the use of *G. mellonella* as a model host to study fungal infections. These authors showed that *G. mellonella* can be used to monitor fungal pathogenicity by a survival assay (Fuchs BB et al 2010).

Larvae of *G. mellonella* at their final instar stage, selected for their weight (330-370 mg) and the absence of cuticle pigmentation in order to limit variability into the study sample, were randomly divided into groups (16 larvae/group) to evaluate PDT efficacy after infection with *C. albicans* (Figure 9).

The reference *C. albicans* strain SC5314 was grown on SDA plates at 30°C for 24 hours. Cells were collected by centrifugation and washed three times with phosphate buffer saline (PBS). Yeast cells were counted using a Burker hemocytometer (Emergo, Landsmeer, The Netherlands) and the yeast cell suspension was properly diluted in sterile distilled water to achieve a final concentration of 5×10^7 cells/ml.

For evaluation of PDT efficacy, 10 μl of a *C. albicans* SC5314 suspension (5×10^5 cells/larva) in saline solution or dyes were inoculated directly into the hemocoel, via the last left pro-leg (Figure 10). Immediately after infection, each larva was treated with laser for the estimated times. Control groups consisted of larvae infected and treated only with laser or with selected dyes. An additional group consisting of untouched larvae served as a control for general viability.



Figure 9. Group of *G. mellonella* larvae; dark color is the main sign of larva death.



Figure 10. Inoculation of *C. albicans* into the *G. mellonella* haemocoel via the last left proleg.

Larvae have been randomly divided into 14 groups as follows:

1. 16 larvae inoculated with 10 μ l of saline solution
2. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in saline
3. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in saline and treated with green laser
4. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 100 μ M erythrosine and treated with green laser
5. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 100 μ M erythrosine
6. 16 larvae inoculated with 10 μ l of 100 μ M erythrosine
7. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in saline and treated with blue laser

8. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 100 μ M curcumin and treated with blue laser
9. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 100 μ M curcumin
10. 16 larvae inoculated with 10 μ l of 100 μ M curcumin
11. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in saline and treated with red laser
12. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 10 μ M toluidine blue and treated with red laser
13. 16 larvae inoculated with 10 μ l of *C. albicans* SC5314 suspension in 10 μ M toluidine blue
14. 16 larvae inoculated with 10 μ l of 10 μ M toluidine blue.

After irradiation larvae were then transferred into clean Petri dishes, one for each experimental group, incubated at 37°C in the dark and monitored for survival daily for 9 days.

Laser irradiation for *G. mellonella* first studies has been performed in continuous mode for the different wavelengths with fluences chosen on the basis of results of *in vitro* study with the following parameters:

- Laser 650 nm red diode was applied for 307 seconds for a fluence of 10 J/cm².
- Laser 405 nm blue diode was applied for 50 seconds for a fluence of 10 J/cm².
- Laser 532 nm green diode was applied for 285 seconds for a fluence of 30 J/cm².

In the second phase of *G. mellonella* studies, after protocol optimization, laser treatment was performed for the 3 different devices at 10 J/cm², or 307 seconds for 650 nm red diode laser, 50 seconds for 405 nm blue diode laser, 95 seconds for 532 nm green diode laser.

Survival curves of treated and control animals were compared by the Mantel-Cox log-rank and Gehan-Breslow-Wilcoxon test using GraphPad Prism 6 statistical software. A value of $p < 0.05$ was considered significant.

All experiments were repeated twice, representative experiments are presented.

Microbial strain and culture conditions for biofilm formation

For this study, *C. albicans* SC5314 was used. The yeast was grown on SDA plates at 30°C for 24 h. Cells were added to RPMI 1640 buffered with MOPS to a concentration of 10⁶ cells/ml, established by McFarland turbidity standard. Cells were cultured on resin discs of polymethyl methacrylate (PMMA) (Paladur, Heraeus, Italia) of 8 mm diameter, placed in 12-wells culture plates (Corning, NY, USA).

For scanning electron microscopy (SEM), 5 ml of yeast cell suspension (10⁶ cells/ml in RPMI 1640) were put onto resin discs within each well and incubated for 18 h at 37°C on an orbital shaker at 180 rpm. Discs were removed 18 h later and washed twice with 0.1 M PBS to remove non-adherent cells.

For XTT assay, *C. albicans* biofilms were grown by dispersing 100 µl/well of standardized cell suspensions in flat bottomed 96-wells microtitre plates. Plates

were incubated 18 h at 37°C on an orbital shaker at 180 rpm and then washed twice as described previously.

Dyes and laser sources for biofilm study

The final working concentration was 100 µM for erythrosine and curcumin, and 10 µM for toluidine blue.

Laser irradiation has been performed in continuous mode for the different wavelengths. Time irradiation was planned for a fluence of 10 J/cm² (307 seconds for red diode laser, 50 seconds for blue diode laser and 95 seconds for green diode laser) (Figure 11).

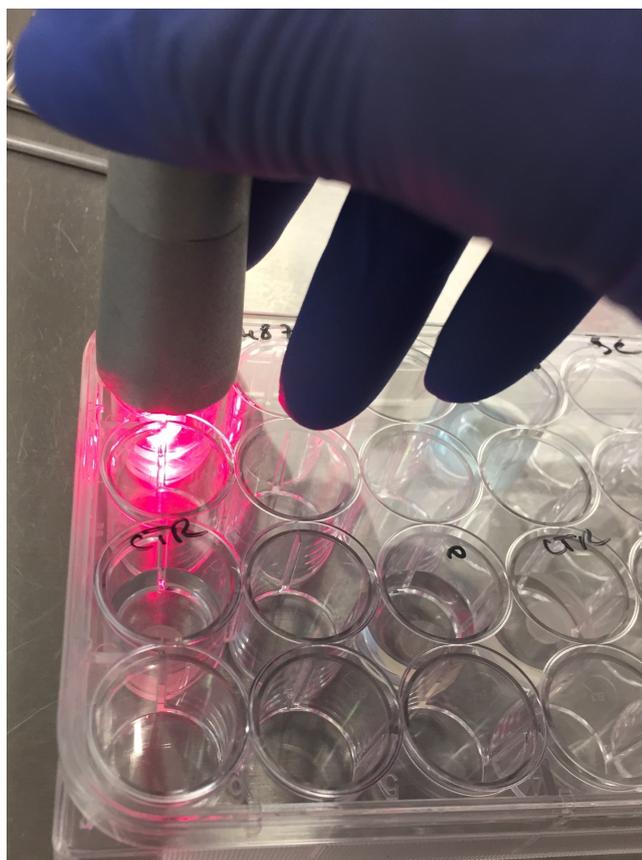


Figure 11. Laser application on resin discs placed in a 24-wells culture plate.

Scanning electron microscopy (SEM)

After PDT protocols application (laser alone or laser with dye), resin discs were placed in fixative (4% v/v formaldehyde in PBS) overnight. Samples were then dehydrated with a series of ethanol (70% for 5 minutes, 95% for 5 minutes and 100% for 10 minutes) and, finally, air-dried in a desiccator.

The disc surfaces were coated with a thin film of gold (Au) in a vacuum evaporator (Ion Sputter, JEOL) (Figure 12) and observed under a scanning electron microscope (JEOL JSM-5310LV, Japan) (Figure 13) in low vacuum mode between 15 and 20 kV. Images were processed for display using SemAforE software (JEOL AB).

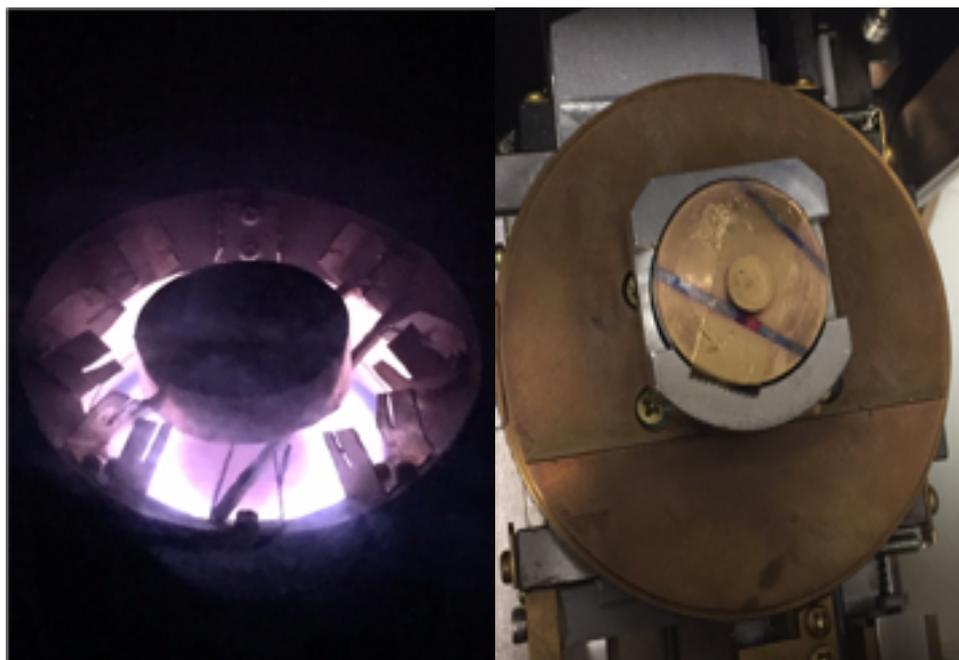


Figure 12. Metallization of the disc surfaces with gold (Au) in a vacuum evaporator (Ion Sputter, JEOL).

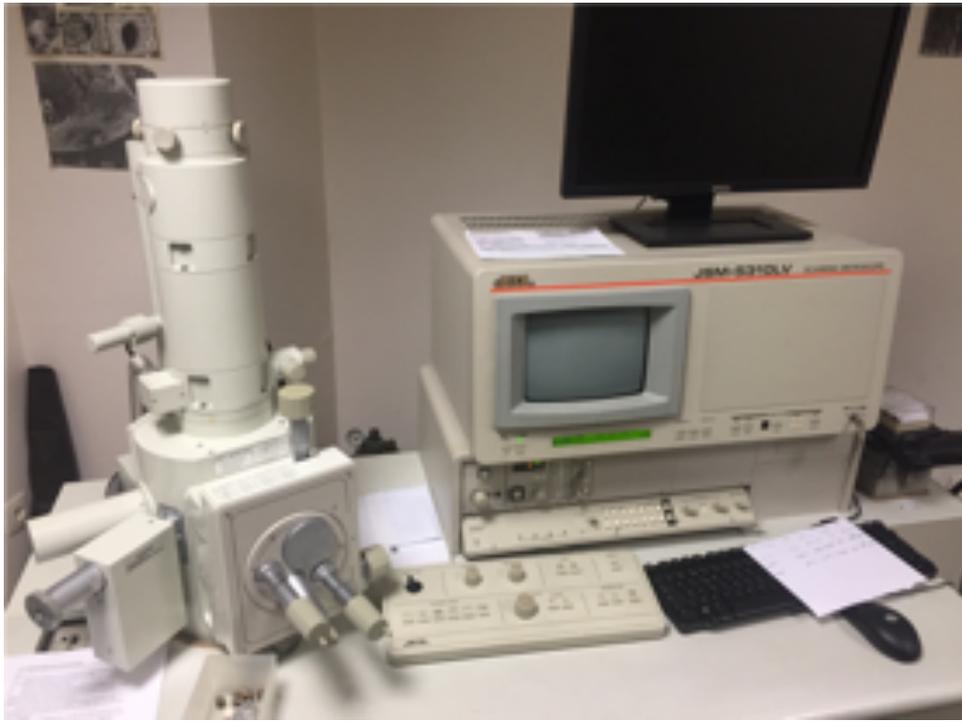


Figure 13. Scanning electron microscope (JEOL JSM-5310LV, Japan) used in low vacuum mode between 15 and 20 kV for samples observation.

Peptide

A previously described synthetic killer decapeptide (KP) endowed with candidacidal activity was used in this study (Manfredi M et al 2007). A stock solution was prepared in DMSO (20 mg/ml) and stored at 4°C until use.

Candidacidal activity of KP

The fungicidal activity of KP, associated or not with PDT protocol applications, on *C. albicans* cells adhered to acrylic discs was evaluated *in vitro* by the XTT assay. Biofilms were grown in 96-wells microtitre plates for 18 h as previously described, then 100 µl of a KP solution (20 µg/ml) in sterile distilled water or sterile water alone (control) were added into the wells. At the same time sterile

water or dyes were added and PDT applications were made to the proper wells. All the plates were left at room temperature for the same time. Microtitre plates were then re-incubated for 2 h at 37°C on an orbital shaker at 180 rpm. Each assay was carried out in triplicate.

XTT assay

After PDT protocols application, associated or not with KP treatment, a semiquantitative measure of biofilm production in each well was assessed using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay (Sigma-Aldrich, USA) (Figure 14). Briefly, a saturated solution of XTT (0.5 mg/ml) was prepared, aliquoted, and stored at -70°C.

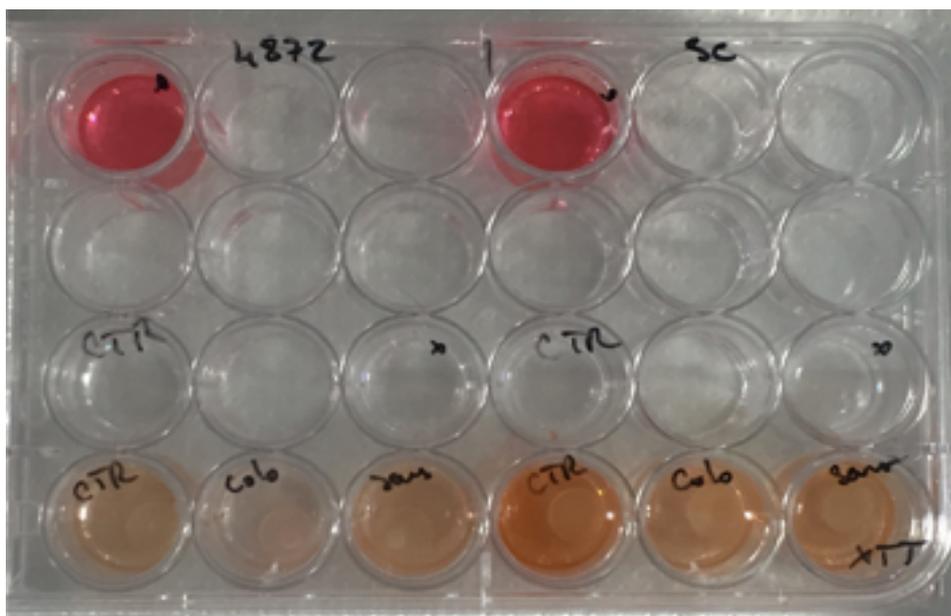


Figure 14. Preparation of XTT assay.

Before XTT was used, 100 μ l of streptavidin were added to 5 ml of XTT solution. Then 50 μ l of the XTT reaction mixture (activation reagent and XTT reagent) prepared according to manufacturer's recommendations were added to each well. The plates were incubated in the dark at 37°C for 2 h and the colorimetric change resulting from XTT reduction, directly correlated to biofilm metabolic activity, was measured in a microtitre plate reader (ELx800, Biotek Instruments, USA) at 490 nm. An inhibitory percentage was calculated by the following formula: $[(\text{control}-\text{treatment})/\text{control}]\times 100$.

Assays were performed in triplicate and three independent experiments were carried out.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software.

Data are reported as the mean \pm standard deviation (SD) from triplicate samples and were evaluated using ANOVA. Multiple comparison analysis was realized with Tukey's test.

A value of $p<0.05$ was considered significant and a value of $p<0.01$ was considered very significant.

Results of the systematic review

The initial search in the Pubmed database resulted in 48 papers. After the analysis of titles and abstracts, 35 studies were selected for screening of the full texts, 13 of the 48 articles were excluded (1 review, 1 comments to a previous article, 1 not on antimicrobial effect, 7 clinical or *in vivo* studies, 1 on LED device, 1 on quantum dots, 1 not on *C. albicans*); 14 articles were excluded after the full text analysis because not on the antimicrobial effect of PDT or using LED devices (Figure 15).

Thus, a total of 21 papers formed the basis of this systematic review. Table 1 summarizes the reason of the exclusion of the articles not included in the review.

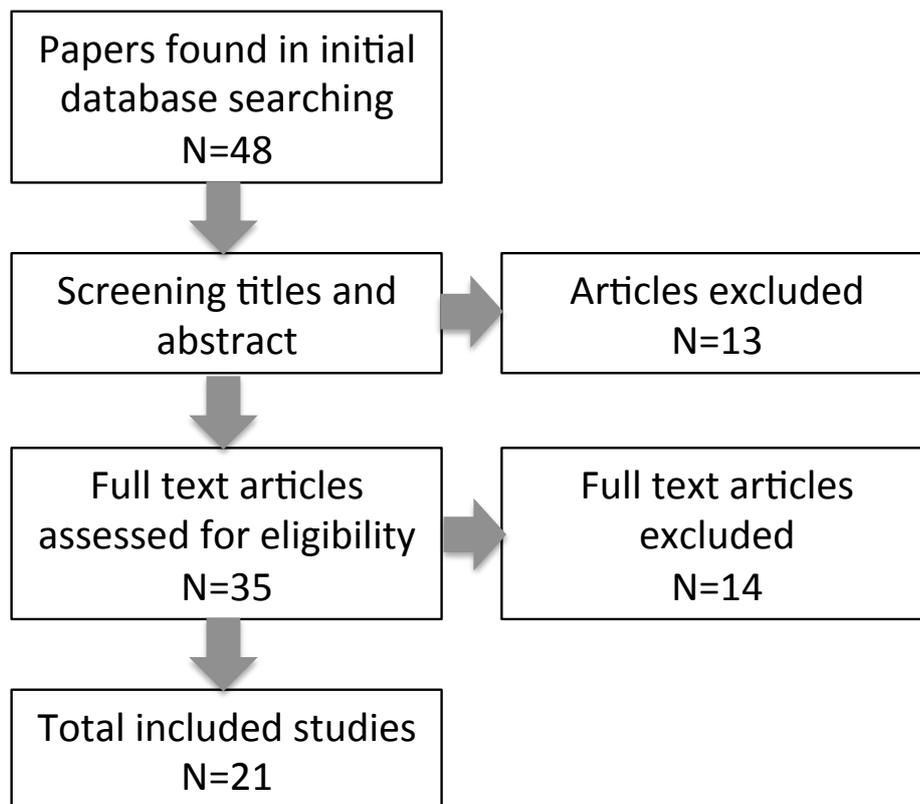


Figure 15. Flowchart of search strategy to identify eligible studies.

Table 1. Reasons of the exclusion of the articles not included in the review.

Reference	Reason of the exclusion
Pavlič A et al 2014	Clinical study
Ferreira LR et al 2016	LED devices
Freire F et al 2016	<i>In vivo</i> study
Silva MP et al 2016	LED devices
Quishida CC et al 2015 a	LED devices
Viana OS et al 2015	Quantum dots
Morton CO et al 2014	Not on <i>C. albicans</i>
Khademi H et al 2014	<i>In vivo</i> study
Grinholc M et al 2015	Comments
Machado-de-Sena RM et al 2014	<i>In vivo</i> study
Javed F et al 2014	Review
Rosseti IB et al 2014	LED devices
Freire F et al 2014	LED devices
Quishida CC et al 2015 b	LED devices
Barbério GS et al 2014	LED devices
Andrade MC et al 2013	LED devices
Ribeiro AP et al 2013	LED devices
Dovigo LN et al 2011	LED devices
Mima EG et al 2011	LED devices
da Silva Martins J et al 2011	<i>In vivo</i> study
Pasychnikova N et al 2009	Full text not available
Junqueira JC et al 2009	<i>In vivo</i> study
Lambrechts SA et al 2005 (a)	Not on <i>C. albicans</i>
Lambrechts SA et al 2005 (b)	Halogen lamp
Lambrechts SA et al 2005 (c)	Halogen lamp
Teichert MC et al 2002	<i>In vivo</i> study
Gibbs NK et al 1988	Not on <i>C. albicans</i> - UVA

The data presented in the selected studies show a large variation on laser parameters. Among the wavelengths used, there was a predominance of visible light (red) spectrum, particularly at 660 nm wavelength, with methylene blue as the most used photosensitizer (used in 15 out of 21 studies).

Table 2 summarizes the main parameters of the lasers used in the articles: energy density or fluence was variable between 3.93 J/cm² (Freire F et al 2015) and 350 J/cm² (Pereira CA et al 2011) performed in all the analyzed studies in a single irradiation.

Most of the included studies highlighted a positive effect for PDT protocols in terms of reduction of colony forming units (CFU), reduction of biofilms, inhibition of germ tube formation or reduction of expression of *C. albicans* enzymes. Only the study of Müller P et al reported a limited effect of PDT on biofilm microbiota.

Table 2. Characteristics of the included studies.

Reference	Wavelength (nm)	Dye	Energy density (J/cm ²)	Culture	Irradiation	Results
Azizi A et al 2016	660-808	MB, ICG	10 J/cm ²	Suspension (plus nystatine or chlorexidine)	Single irradiation	Laser application plus ICG caused a significant reduction in <i>C. albicans</i> CFUs
De Oliveira BP et al 2015	660	NaCl or NaOCl	8 J, 90 sec, 600 micrometers	Suspensions with bacteria and NaCl or NaOCl	Single irradiation (in the root canal)	The association of 5.25% NaOCl with PDT was the most effective treatment
Freire F et al 2015	660	MB	3.93 J/cm ²	Biofilms	Single irradiation	PDT with MB showed a slight reduction on the expression of hydrolytic enzymes of <i>C. albicans</i> , without statistical significance.
Nunez SC et al 2015	645	MB	18, 36 and 54 J/cm ²	Suspensions with urea	Single irradiation	Urea stabilizes solution monomers of MB allowing more efficient APDT on <i>C. albicans</i> and it is likely that this observation is valid for other PSs as well.
Xhevdet A et al 2016	660	HELBO Endo blue	6, 18 and 30 J/cm ²	Suspensions with bacteria and NaOCl	Single irradiation	Percent of dead cells in treatment groups were significantly higher compared to control group
Fekrazad R et al 2015	810	ICG, EO	55 J/cm ²	Suspensions with bacteria	Single irradiation	Cell reduction rates (%) in <i>C. albicans</i> groups were 99.99 (CE), 91.67 (IRLE), 86.67 (CC), 72.37 (E) and 67.27 (RL)

Reference	Wavelength (nm)	Dye	Energy density (J/cm ²)	Culture	Irradiation	Results
Fekrazad R et al 2015	810-630	NMB-EMUNDO	55 J/cm ²	Suspensions	Single irradiation	APDT with either EmunDo® or new methylene blue (NMB) considerably diminished the viability of inoculated <i>C. albicans</i> ($p < 0.001$) by log reduction of 1.9 and 3.37, respectively, compared with the control group.
Sabino CP et al 2015	660	MB	2.5 W/cm ² 10 times/min	Biofilm in root canal	Single irradiation	APDT showed to be an effective way to inactivate <i>C. albicans</i> biofilms. PDT exerted a fungicidal effect on biofilms of <i>C. albicans</i> serotypes A and B; serotype B was more sensitive than serotype A.
Rossoni RD et al 2014	660	MB	26.3 J/cm ²	Biofilm	Single irradiation	Using CTAB-liposomes as PS nanocarriers is valuable not only in reducing the concentration of PSs required to induce a PDT effect, but also in enhancing the overall antimicrobial efficacy of the PSs. Significant reduction observed in cells exposed to 18 and 27 J/cm ² , ability to form germ tubes significantly decreased after exposition to sublethal APDI
Yang YT et al 2013	662	Ce6	50 J/cm ²	Suspensions	Single irradiation	
Kato IT et al 2013	660	MB	9, 18 and 27 J/cm ²	Suspensions	Single irradiation	

Reference	Wavelength (nm)	Dye	Energy density (J/cm ²)	Culture	Irradiation	Results
Khan S et al 2012	660	MB with and without nanoparticles	38.2 J/cm ²	Biofilms	Single irradiation	Antibiofilm assays and microscopic studies showed significant reduction of biofilm and adverse effect against <i>Candida</i> cells in the presence of conjugate.
Junqueira JC et al 2012	660	ZnPc	26.3 J/cm ²	Biofilms	Single irradiation	All biofilms studied were susceptible to PDI with statistically significant differences. The strains of <i>Candida</i> genus were more resistant to PDI than emerging pathogens <i>Trichosporon mucoides</i> and <i>Kodamaea ohmeri</i> .
Pupo YM et al 2011	660	TB, MB	53 J/cm ²	Suspensions	Single irradiation	The number of viable <i>C. albicans</i> cells was reduced significantly after PDT using MB or mainly TB associated to diode laser irradiation.
Queiroga AS et al 2011	660	MB	60, 120 and 180 J/cm ²	Suspensions	Single irradiation	The three evaluated doses determined meaningful inactivation of <i>Candida</i> spp. with 180J/cm ² as most effective dose, inactivating 78% of CFU/ml.
Mitra S et al 2011	514	TMP-1363, MB	90 J/cm ²	Suspensions	Single irradiation	Photosensitization with TMP-1363 resulted in a greater than three-log increase in killing of <i>C. albicans in vitro</i> compared to MB.

Reference	Wavelength (nm)	Dye	Energy density (J/cm ²)	Culture	Irradiation	Results
Pereira CA et al 2011	660	MB	350 J/cm ²	Biofilms	Single irradiation	Significant decreases in the viability of all microorganisms were observed for biofilms exposed to PDI mediated by MB dye.
Souza RC et al 2010	660	MB, TB, MG	15.8, 26.3 and 39.5 J/cm ²	Suspensions	Single irradiation	The number of CFU/ml was reduced by between 0.54 log(10) and 3.07 log(10) and depended on the laser energy density used. TB, MB and MG were effective photosensitizers in antimicrobial photodynamic therapy against <i>C. albicans</i> , as was low-power laser irradiation alone. Combination of MB and laser promoted a decrease in <i>Candida</i> growth more pronounced in the presence of 0.05 mg/ml MB and with an energy density of 28 J/cm ² and with an association with an increase in membrane permeabilization.
Giroldo LM et al 2009	684	MB	28 J/cm ²	Suspensions	Single irradiation	Inhibition in both germ tube and filament formation occur only after phototoxic response is started by appropriated combination of light and photosensitizer.
Munin E et al 2007	683	MB	28 J/cm ²	Suspensions	Single irradiation	

Reference	Wavelength (nm)	Dye	Energy density (J/cm ²)	Culture	Irradiation	Results
Müller P et al 2007	665	MB	Not clear, 75 mW for 60 sec	Biofilms	Single irradiation	Hypochlorite at 0.5% and 5% concentration exhibited a significantly ($P = 0.05$) increased antimicrobial potential compared with 0.2% chlorhexidine, gasiforme ozone, and PDT, which reduced the microbiota of the biofilm by less than one order of magnitude.

MB: methylene blue; ICG: indocyanine green; EO: Citrus aurantifolia essential oil; NMB: EmunDo® or new methylene blue; TB: Toluidine blue; Ce6: Chlorine e6 encapsulated in cationic CTAB-liposomes; CUR: Curcumin; ZNPC: cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H,31H-phthalocyanine; TMP-1363: meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate; MG: malachite green; CE: natural and tungsten lights, IRLE: Indocyanine green (EmunDo, 1 mg/ml), CC: control non-activated essential oil, E: indocyanine green alone; IRL: Laser alone.

Results

No inhibition of growth was obtained with all lasers on fungal suspensions in saline solutions at any fluence value. Results for fungal suspensions in the presence of dyes are summarized in Tables 3-5.

Red diode laser used with toluidine blue caused a growth inhibition variable between 79.31% (for fluence of 10 J/cm²) and 95.79% for fluence of 20 and 30 J/cm².

The maximum inhibition of growth (100%) was obtained with the blue diode and curcumin at any used fluence.

Green diode laser used with erythrosine caused a growth inhibition variable between 10.34% (for fluence of 10 J/cm²) and 39.85% for fluence of 30 J/cm².

We did not record any inhibition growth on solid culture medium without dye.

For culture plates with dye, inhibition areas were visible for plates with curcumin and erythrosine: the zone of inhibition on the plates with curcumin had a diameter respectively of 6.38 ± 0.6 mm, 8.51 ± 0 mm and 8.51 ± 0 mm for the fluences of 10, 20 and 30 J/cm² (Figure 16); the zone of inhibition on the plates with erythrosine had a diameter respectively of 10.2 ± 0 mm, 11.9 ± 0 mm and 11.9 ± 0 mm for the fluences of 10, 20 and 30 J/cm² (Figure 17).

Table 3. Effect of red laser (650 nm) application on *C. albicans* suspensions with and without toluidine blue.

Samples		Mean CFU	SD	% inhibition vs control
Control		261	5	-
Red diode without toluidine blue	Fluence 1	331	2	-26.82
	Fluence 2	307	40	-17.62
	Fluence 3	311	2	-19.16
Toluidine blue 10 μ M		62	13	76.25**
Red diode with toluidine blue	Fluence 1	54	74	79.31*
	Fluence 2	11	13	95.79***
	Fluence 3	11	13	95.79***

CFU, colony forming unit; SD, standard deviation; Fluence 1: 10 J/cm²; Fluence 2: 20 J/cm²; Fluence 3: 30 J/cm²; * p <0.05; ** p <0.01; *** p <0.001 vs control assessed by Student's *t*-test

Table 4. Effect of blue laser (405 nm) application on *C. albicans* suspensions with and without curcumin.

Samples		Mean CFU	SD	% inhibition vs control
Control		261	5	-
Blue diode without curcumin	Fluence 1	262	11	-0.38
	Fluence 2	255	5	2.30
	Fluence 3	259	20	0.77
Curcumin 100 μ M		219	11	16.09*
Blue diode with curcumin	Fluence 1	0	0	100***
	Fluence 2	0	0	100***
	Fluence 3	0	0	100***

CFU, colony forming unit; SD, standard deviation; Fluence 1: 10 J/cm²; Fluence 2: 20 J/cm²; Fluence 3: 30 J/cm²; * p <0.05; *** p <0.001 vs control assessed by Student's *t*-test

Table 5. Effect of green laser (532 nm) application on *C. albicans* suspensions with and without erythrosine.

Samples		Mean CFU	SD	% inhibition vs control
Control		261	5	-
Green diode without Erythrosine	Fluence 1	259	50	0.77
	Fluence 2	292	23	-11.88
	Fluence 3	287	12	-9.96
Erythrosine 100 μ M		305	49	-16.86
Green diode with Erythrosine	Fluence 1	234	52	10.34
	Fluence 2	237	21	9.20
	Fluence 3	157	67	39.85

CFU, colony forming unit; SD, standard deviation; Fluence 1: 10 J/cm²; Fluence 2: 20 J/cm²; Fluence 3: 30 J/cm²

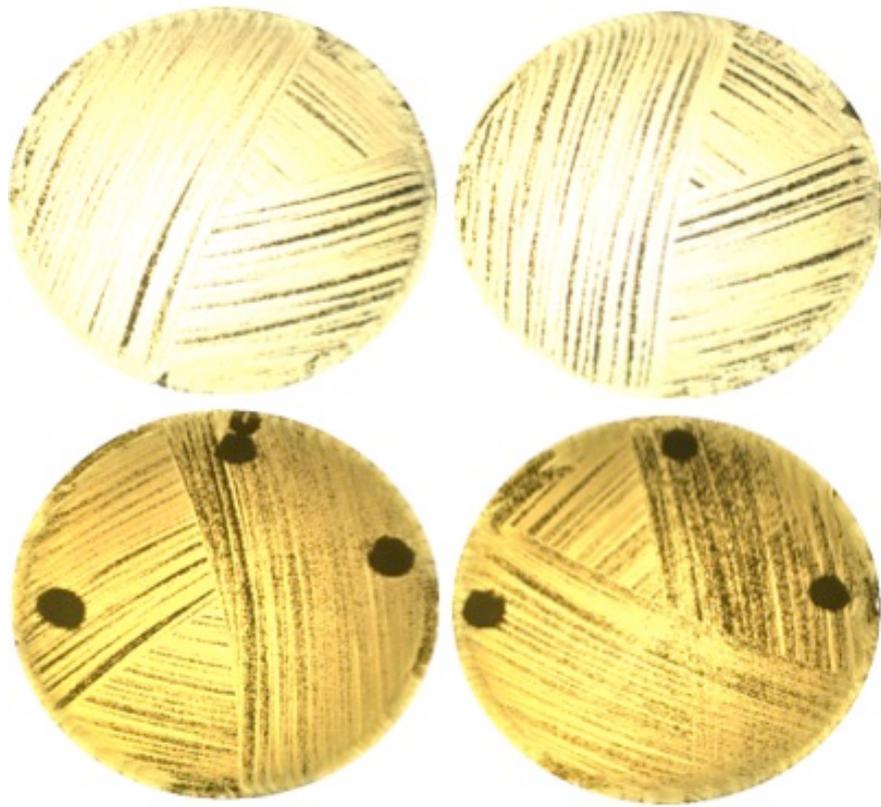


Figure 16. Plates without (upper) and with (lower) curcumin.

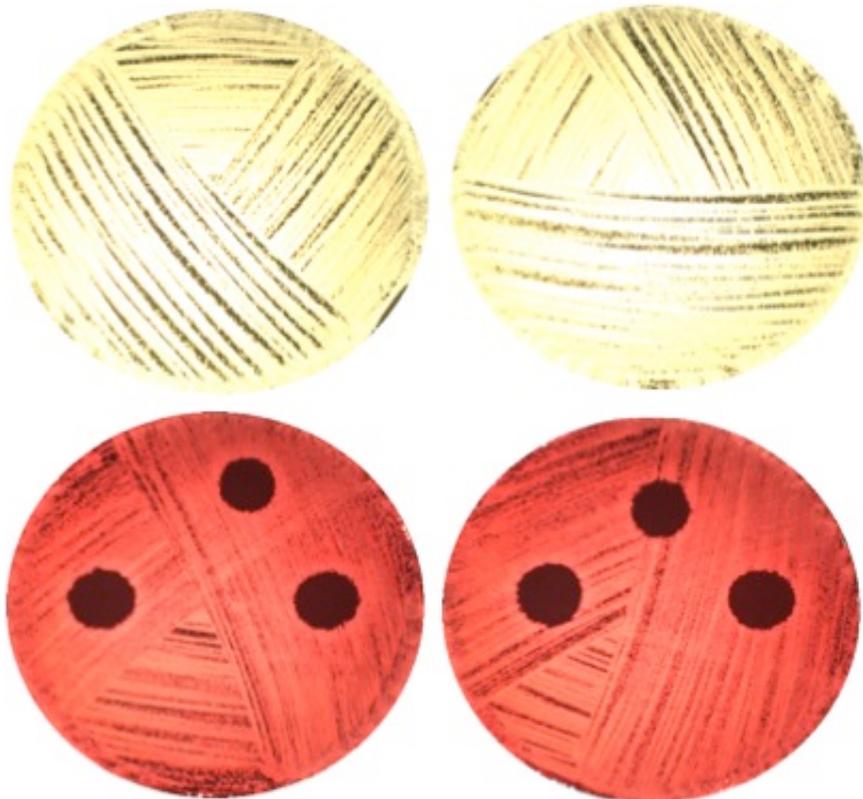


Figure 17. Plates without (upper) and with (lower) erythrosine.

Evaluation of potential *in vivo* toxicity of dyes

No statistically significant difference of survival was observed between *G. mellonella* groups treated with saline solution or dyes (Figure 18).

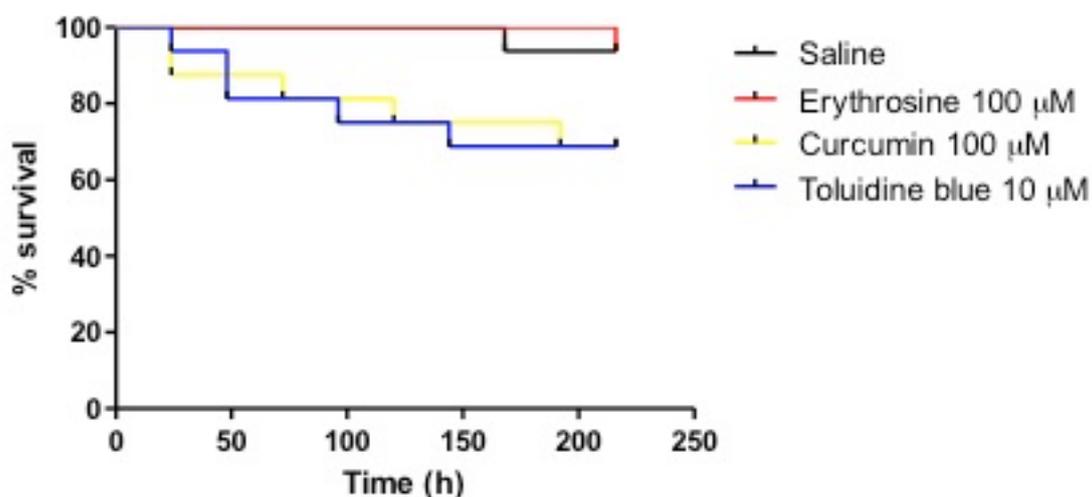


Figure 18. *In vivo* evaluation of toxicity for the different dyes at selected concentration.

Evaluation of PDT protocols in *Galleria mellonella*

The comparison of survival curves of *G. mellonella* infected with *C. albicans* treated with different dyes did not show any statistically significant difference (Figure 19) as well as the comparison of survival curves of *G. mellonella* infected with *C. albicans* treated with different lasers without dye (Figure 20) and treated with different dyes and lasers (Figure 21).

No statistical difference in terms of survival rate was detected for laser used without dyes and for laser used with curcumin and erythrosine.

A statistically significant difference was found between *G. mellonella* infected with *C. albicans* treated with toluidine blue and red laser compared with *G. mellonella* infected with *C. albicans* treated with only toluidine blue or only red laser ($p < 0.05$) (Figure 22).

In any case, difference was not statistically significant in comparison to *G. mellonella* infected with *C. albicans* and not treated.

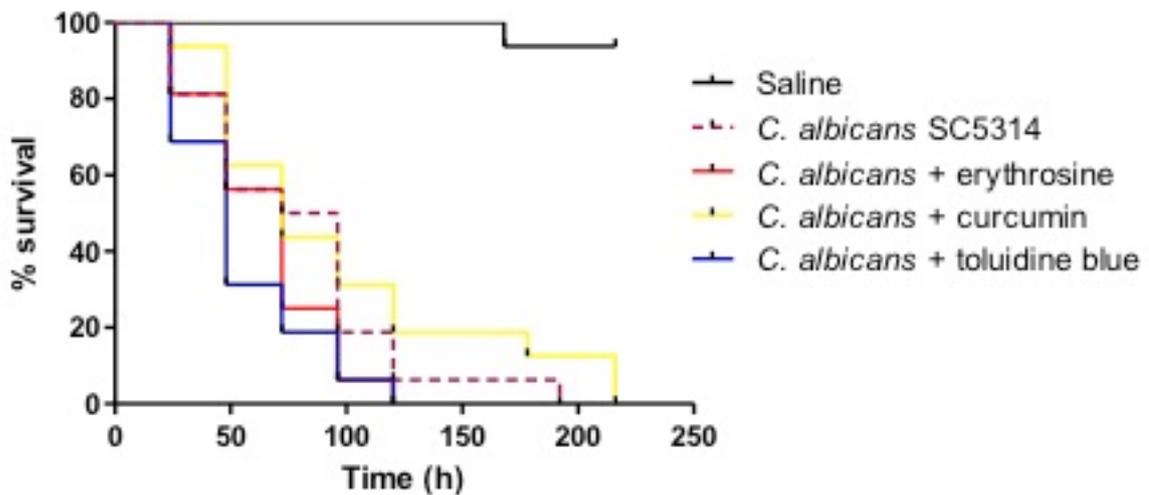


Figure 19. Survival curves of *G. mellonella* larvae infected with *C. albicans* and treated with the 3 dyes without laser.

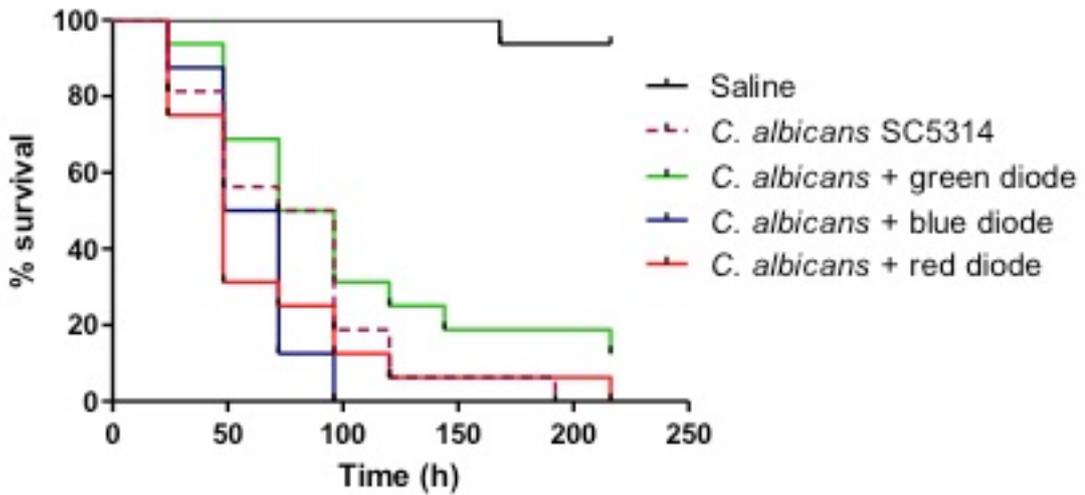


Figure 20. Survival curves of *G. mellonella* larvae infected with *C. albicans* and treated with the 3 lasers without dye.

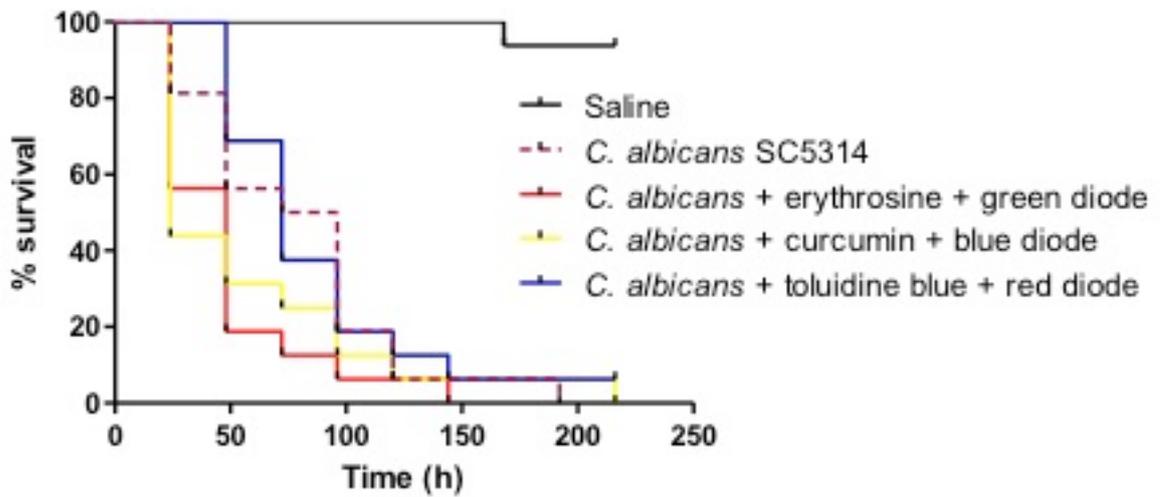


Figure 21. Survival curves of *G. mellonella* larvae infected with *C. albicans* and treated with the 3 lasers with dye.

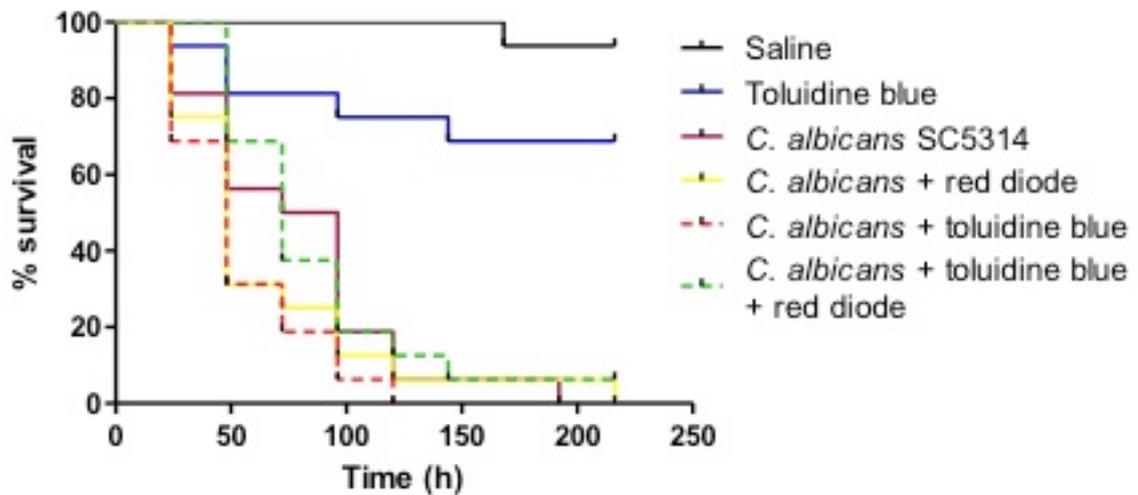


Figure 22 Survival curves of *G. mellonella* larvae treated with saline solution and toluidine blue, infected with *C. albicans* and treated or not with red diode and/or toluidine blue dye.

After optimization of *G. mellonella* model for PDT studies we repeated the same protocol of PDT.

In this second phase of the study on *G. mellonella* larvae infected with *C. albicans* SC5314, the every performed treatment (laser alone, dye alone or combinations of laser and dye) led to a significant increase in survival in comparison to infected animals inoculated with saline ($p < 0.001$) (Figures 23-25).

The combination of toluidine blue and red diode laser application led to a prolonged survival compared to dye alone or laser application alone, although the difference in survival was not statistically significant between the 3 groups (Figure 26).

A statistically significant difference ($p=0.02$) in survival was found between the group inoculated with curcumin alone compared to the group treated with blue diode laser coupled with curcumin (Figure 27).

A statistically significant difference ($p=0.03$) in survival was found between the group of larvae irradiated with green diode laser compared to the group treated with laser coupled with erythrosine (Figure 28).

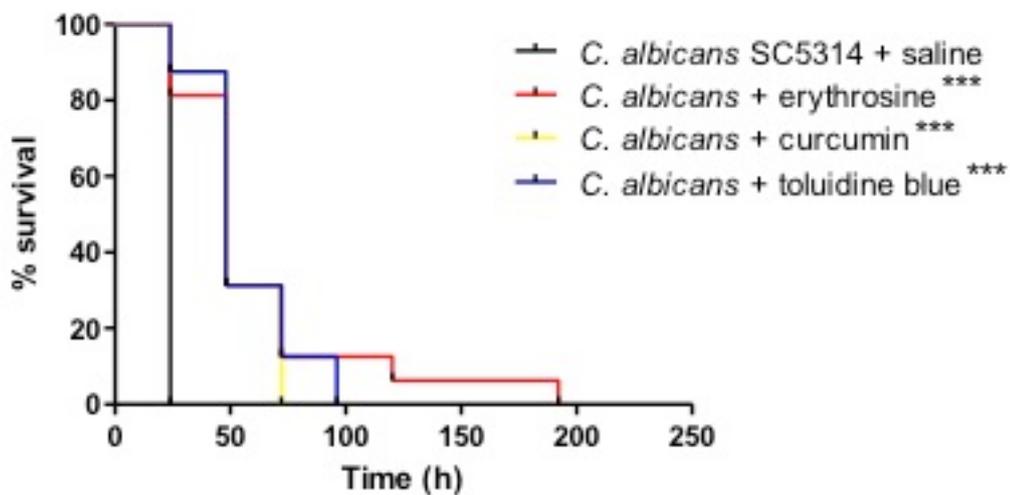


Figure 23. Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) in saline (control) or dye solutions (16 animals/group). *** significant difference in survival in comparison to the control group ($p < 0.001$) as assessed by the Mantel-Cox log-rank test. No significant difference in survival was observed between the 3 groups of animals inoculated with dye solutions.

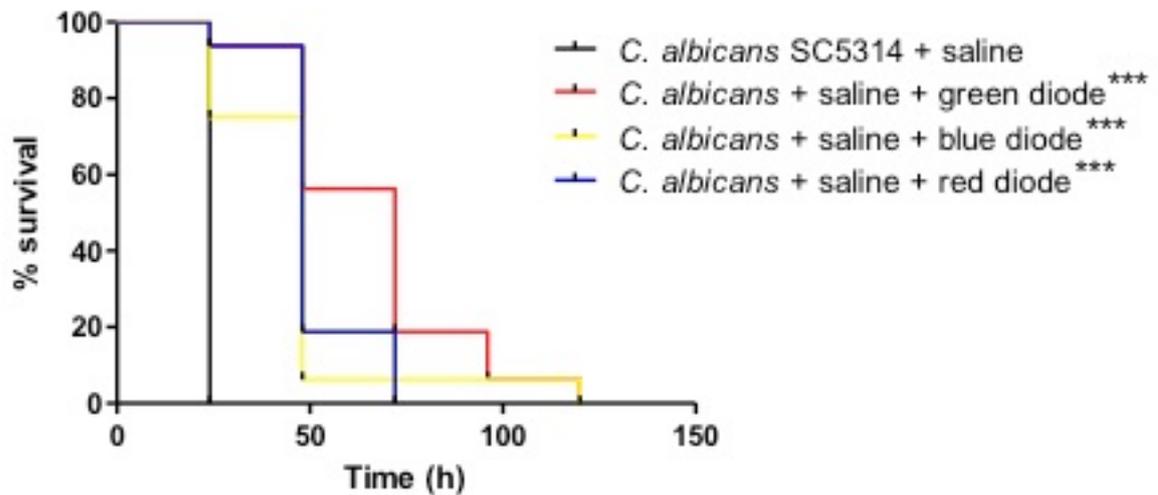


Figure 24. Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) and exposed to light irradiation (16 animals/group). *** significant difference in survival in comparison to the control group ($p < 0.001$) as assessed by the Mantel-Cox log-rank test. No significant difference in survival was observed between the 3 groups of animals exposed to light irradiation.

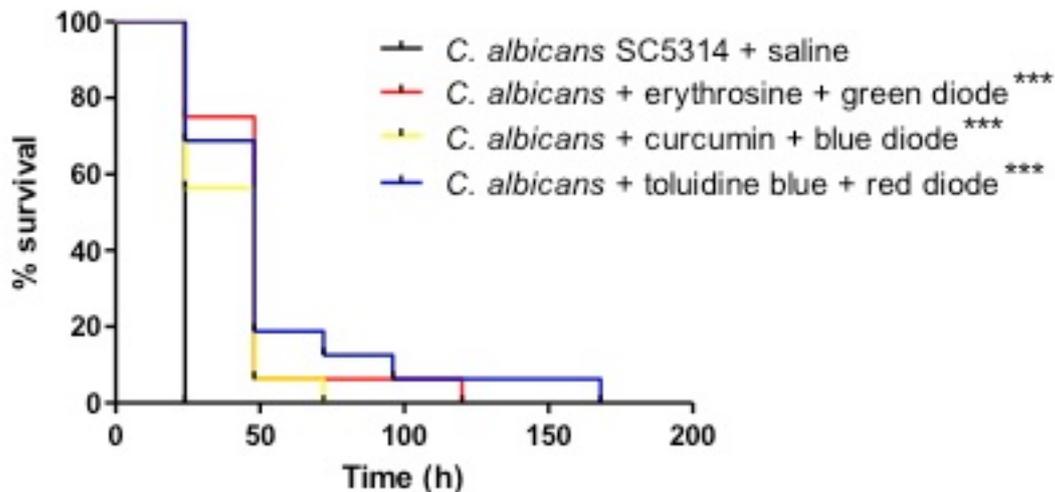


Figure 25. Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) in saline (control) or dye solution and exposed to light irradiation (16 animals/group). *** significant difference in survival in comparison to the control group ($p < 0.001$) as assessed by the Mantel-Cox log-rank test. No significant difference in survival was observed between the 3 groups of animals exposed to light irradiation.

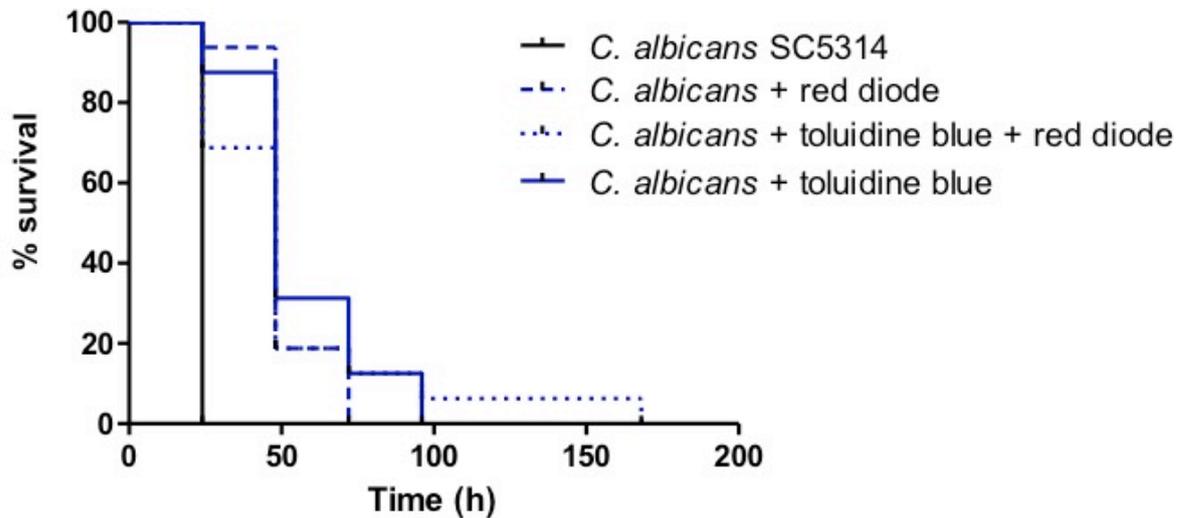


Figure 26: Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) in saline (control) or dye solution and treated with red diode laser alone, toluidine blue alone or red diode laser and toluidine blue (16 animals/group). No significant difference in survival was observed between the 3 groups of treated animals.

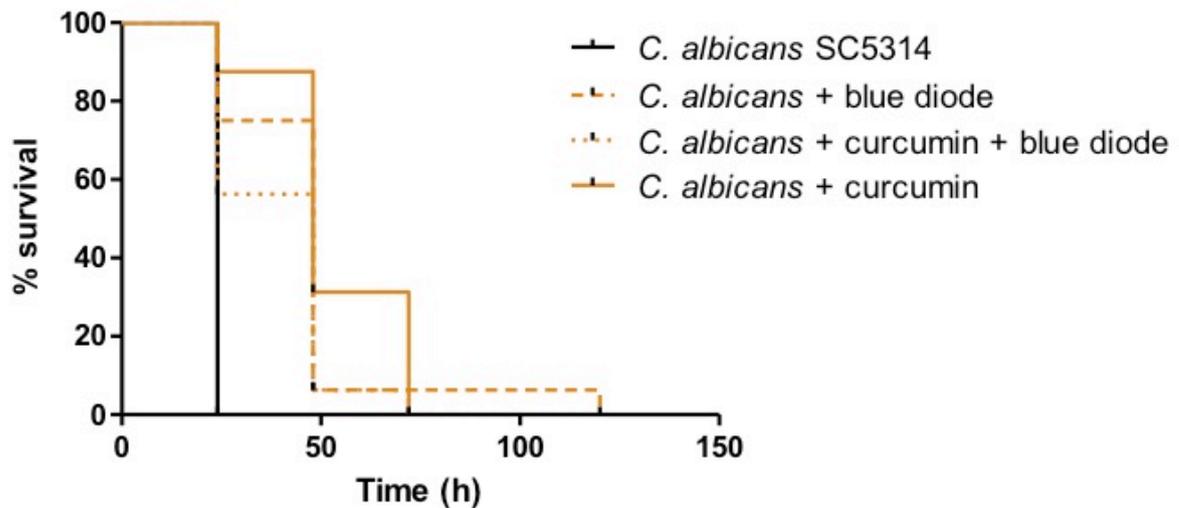


Figure 27: Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) in saline (control) or dye solution and treated with blue diode laser alone, curcumin alone or blue diode laser and curcumin (16 animals/group). A statistically significant difference ($p=0.02$) in survival was found between the group inoculated with curcumin alone compared to the group treated with blue diode laser coupled with curcumin.

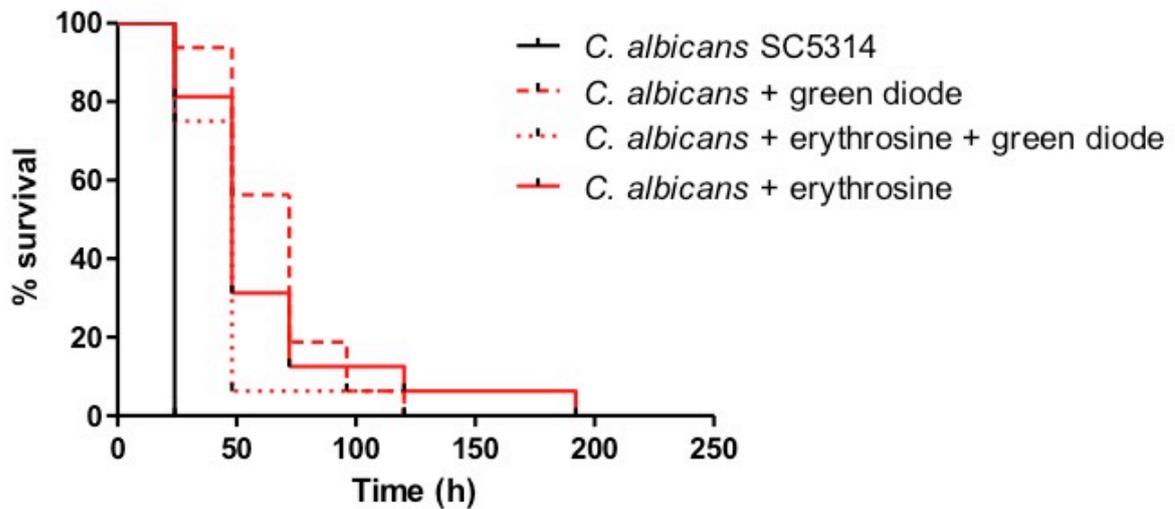


Figure 28: Survival curves of *G. mellonella* larvae infected with *C. albicans* (5×10^5 cells/larva) in saline (control) or dye solution and treated with green diode laser alone, erythrosine alone or green diode laser and erythrosine (16 animals/group). A statistically significant difference ($p=0.03$) in survival was found between the group of larvae irradiated with green diode laser compared to the group treated with laser coupled with erythrosine.

SEM evaluation

The effects of various treatments on *C. albicans* biofilm, in comparison to untreated control (Figure 29) are shown in Figures 30-35.

In comparison to the untreated control, the images show that the most effective treatment was the association of blue diode laser and curcumin (Figure 33), while application of blue diode laser alone (Figure 30) had no effect.

The same images comparison shows that the worst effective treatment was the treatment performed with green diode laser without erythrosine (Figure 34) or with erythrosine (Figure 35).

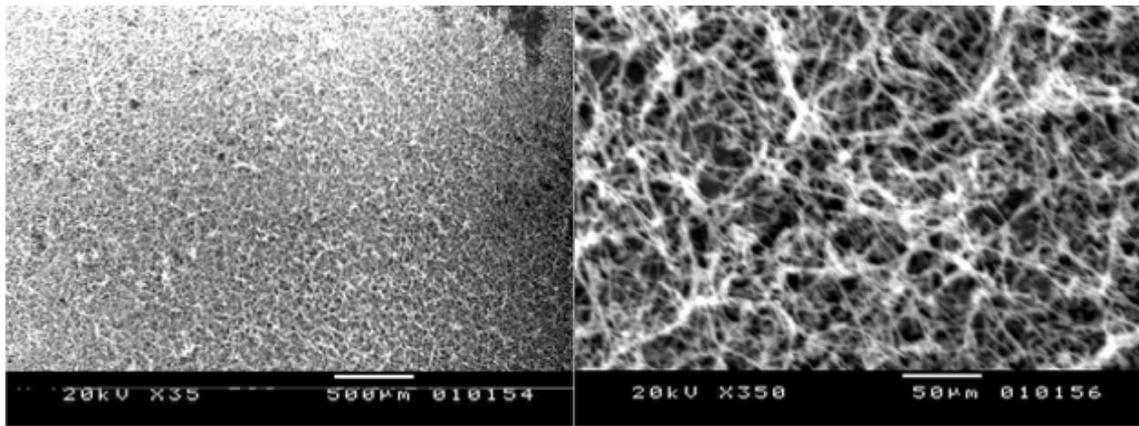


Figure 29. Scanning electron microscopy of *C. albicans* SC5314 biofilm (untreated control). Magnification $\times 35$ (left) and $\times 350$ (right). Images show the presence of a homogeneous biofilm on the surface of the resin disk (left) and the well-organized structure of the biofilm (right).

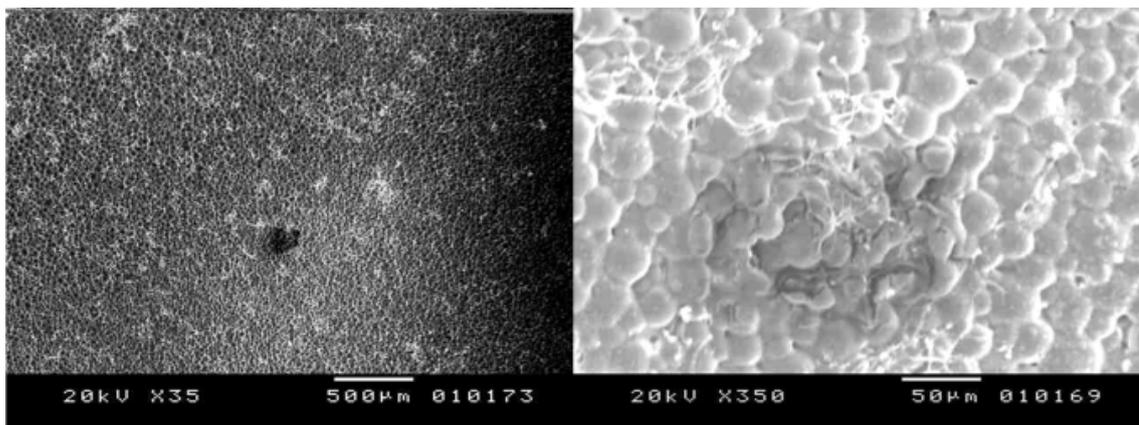


Figure 30. Scanning electron microscopy of *C. albicans* SC5314 biofilm treated with red diode laser (650 nm) without toluidine blue. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the absence of a homogeneous biofilm with scattered patches of fungal structures (left) and the well visible surface of the resin disk not covered by biofilm with rare candidal hyphae (right).

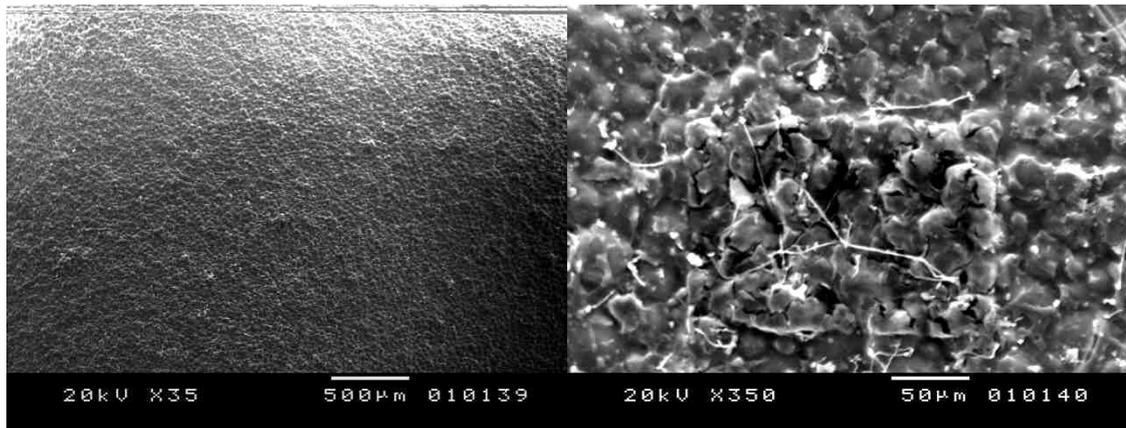


Figure 31. Scanning electron microscopy of *C. albicans* SC5314 biofilm treated with red diode laser (650 nm) with toluidine blue. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the absence of biofilm and isolated fungal structures (left) and the well visible surface of the resin disk not covered by biofilm with rare candidal hyphae (right).

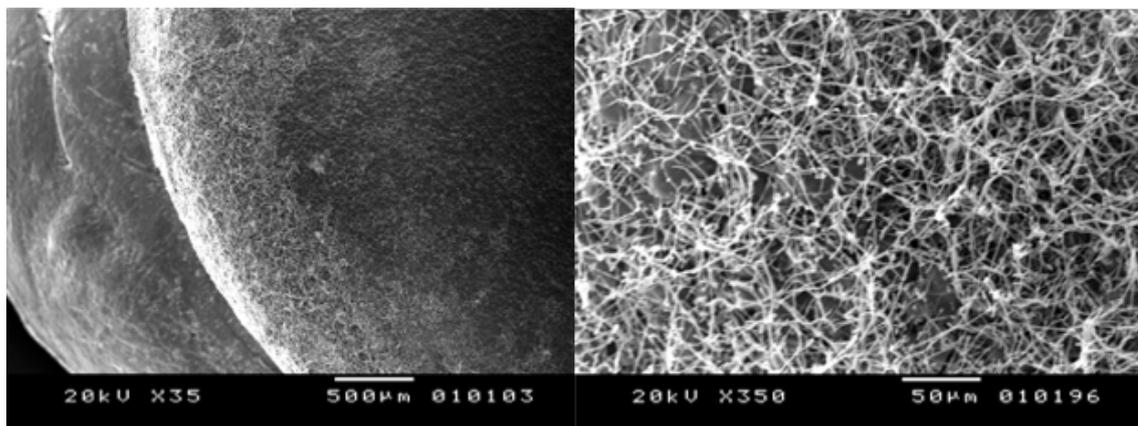


Figure 32. Scanning electron microscopy of *C. albicans* SC5314 biofilm treated with blue diode laser (405 nm) without curcumin. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the presence of a mature biofilm on the surface of the resin disk (left) and the well-organized structure of the biofilm (right).

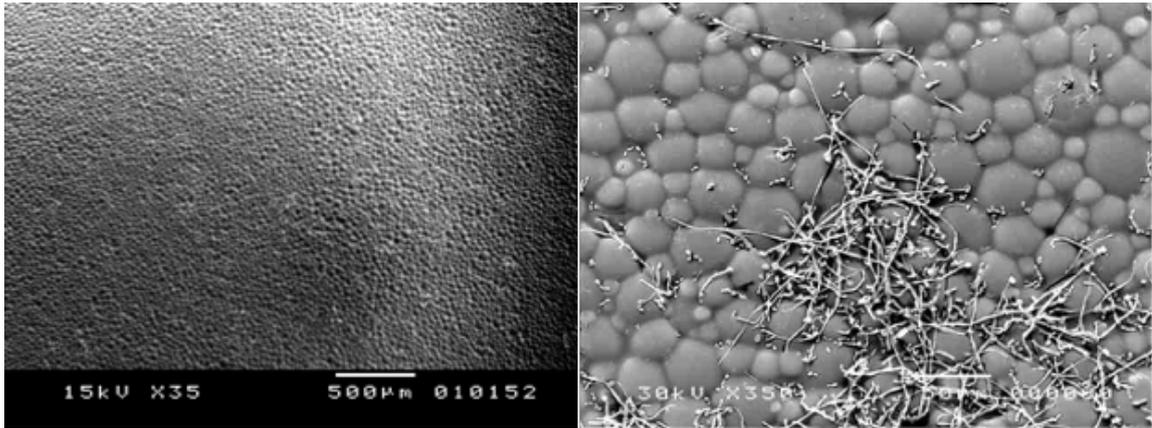


Figure 33. Scanning electron microscopy of *C. albicans* SC5314 biofilm treated with blue diode laser (405 nm) with curcumin. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the absence of biofilm (left) and a group of candidal hyphae and pseudohyphae not covering the surface of the resin disk which is well visible in its morphology (right).

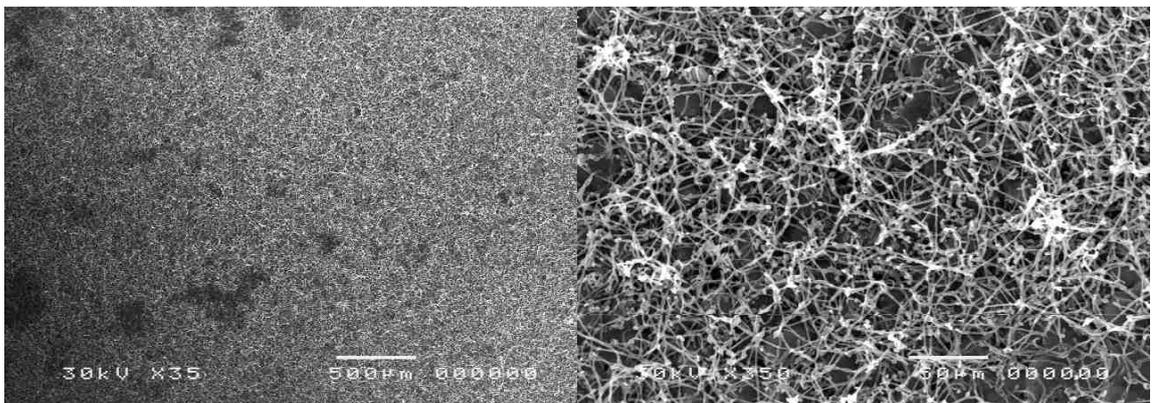


Figure 34. Scanning electron microscopy of *C. albicans* SC5314 biofilm treated with green diode laser (532 nm) without erythrosine. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the presence of a homogeneous biofilm on the surface of the resin disk (left) and the well-organized structure of the biofilm (right).

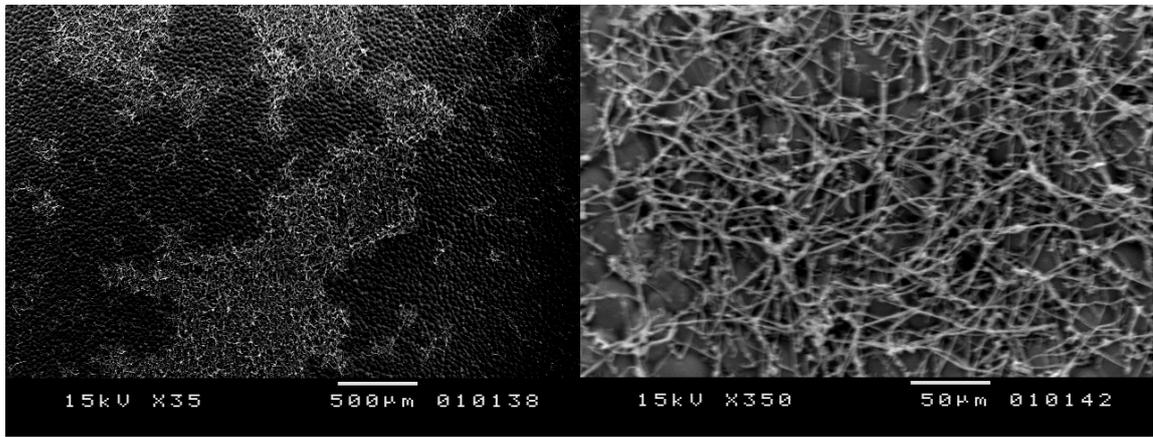


Figure 35. Scanning electron microscopy of C. albicans SC5314 biofilm treated with green diode laser (532 nm) with erythrosine. Magnification $\times 35$ (left) and $\times 350$ (right). Images show the presence of a not completely homogeneous biofilm on the surface of the resin disk (left) and a well-organized structure of the biofilm (right).

XTT assay

The results of the treatment of *C. albicans* biofilm with dyes alone or PDT protocol applications (laser alone or laser with dye) are shown in Table 6 and Figures 36 and 37.

Curcumin was the most effective dye, when used without laser, and showed a significant effect on *C. albicans* biofilm in comparison with the untreated control ($p<0.0001$), but also in comparison with toluidine blue and erythrosine ($p<0.0001$) (Figure 38).

Comparing laser treatments performed without dyes with the untreated control, the best results were obtained with green diode laser (532 nm) ($p=0.0021$) (Figure 39), while the best result for the combination of laser and dye was obtained with blue diode laser (405 nm) and curcumin ($p=0.0005$) (Figure 40).

Treatment performed with red diode laser and toluidine blue did not show any effect on *C. albicans* biofilm, as was for red diode laser alone and toluidine blue alone (Figure 41); conversely, good results were found for green diode laser (532 nm) and erythrosine, with the maximal effect obtained with the combination of laser and dye ($p=0.0068$), but a significant result also for laser alone ($p=0.0131$) (Figure 42). As already said, the best results were obtained with the association of blue diode laser and curcumin with a very significant result in comparison with untreated control ($p<0.0001$), while curcumin alone showed better results than laser alone ($p=0.0057$) (Figure 43).

Table 6. Effects on *Candida albicans* biofilm of treatment with different combinations of laser and dye.

Treatment	OD mean	SD	% Inhibition
Control (no treatment)	2.207	0.152	-
Only TB	2.270	0.035	- 2.85
Red diode laser with TB	2.284	0.082	- 3.49
Red diode laser without TB	2.144	0.085	2.18
Only CUR	1.209	0.062	45.22
Blue diode laser with CUR	1.185	0.15	46.31
Blue diode laser without CUR	1.738	0.15	21.25
Only ERY	1.937	0.045	12.23
Green diode laser with ERY	1.578	0.277	28.50
Green diode laser without ERY	1.647	0.077	25.37

Results of the XTT assay are expressed as mean values and standard deviation (SD) of the optical density (OD) at 490 nm, and percentage of inhibition. TB, toluidine blue; CUR, curcumin; ERY, erythrosine.

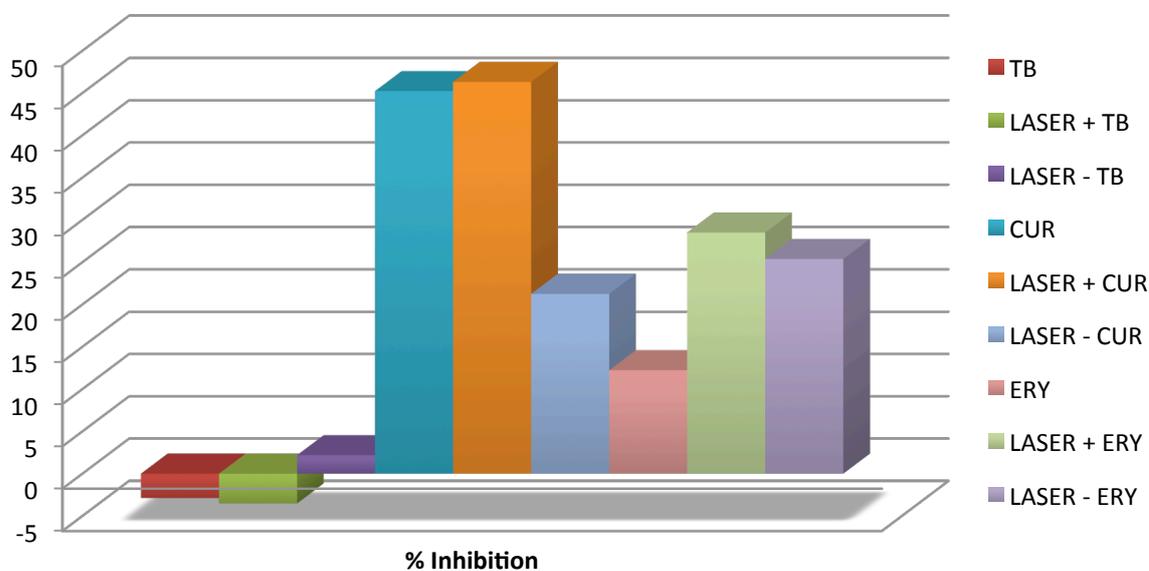


Figure 36. Percentage of inhibition of *C. albicans* SC5314 biofilm after different treatments. Laser + TB, Laser - TB, Red diode laser with or without toluidine blue; Laser + CUR, Laser - CUR, Blue diode laser with or without curcumin; Laser + ERY, Laser - ERY, Green diode laser with or without erythrosine.

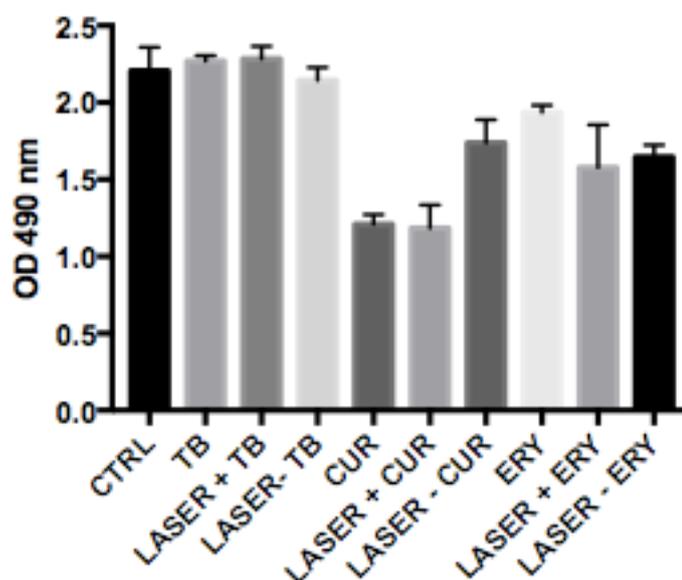


Figure 37. Effects of treatment with different combinations of laser and dye on *C. albicans* biofilm viability. CTRL, untreated control; TB, toluidine blue; CUR, curcumin; ERY, erythrosine; Laser + TB, Laser - TB, Red diode laser with or without TB; Laser + CUR, Laser - CUR, Blue diode laser with or without CUR; Laser + ERY, Laser - ERY, Green diode laser with or without ERY.

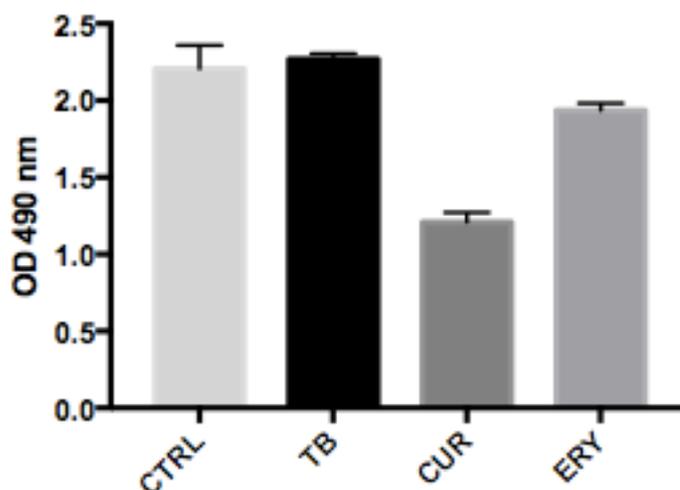


Figure 38. Effects of treatment with dye alone on *C. albicans* biofilm (TB, toluidine blue; CUR, curcumin; ERY, erythrosine). ANOVA analysis revealed a statistical significance ($p < 0.001$) among the untreated control (CTRL) and dyes; Tukey's test found a very significant difference between curcumin and control and between curcumin and the other dyes ($p < 0.0001$) and a statistical significance between erythrosine and control ($p = 0.0216$) and erythrosine and toluidine blue ($p = 0.0068$).

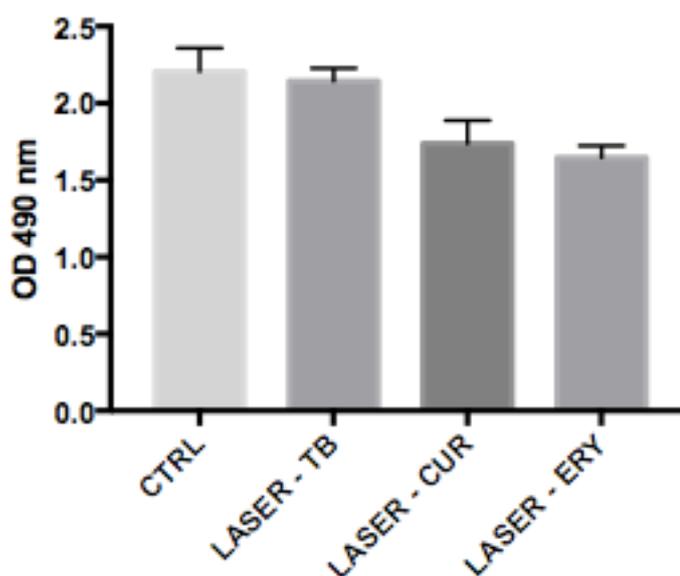


Figure 39. Effects of treatment with laser alone on *C. albicans* biofilm (Laser - TB, Red diode laser without toluidine blue; Laser - CUR, Blue diode laser without curcumin; Laser - ERY,

Green diode laser without erythrosine). ANOVA analysis revealed a statistical significance among the untreated control (CTRL) and groups treated with laser alone ($p=0.0009$); Tukey's test found a statistical significance between untreated control and blue diode laser ($p=0.0064$), between untreated control and green diode laser ($p=0.0021$), between red diode laser and green diode laser ($p=0.0045$) and between red diode laser and blue diode laser ($p=0.0145$).

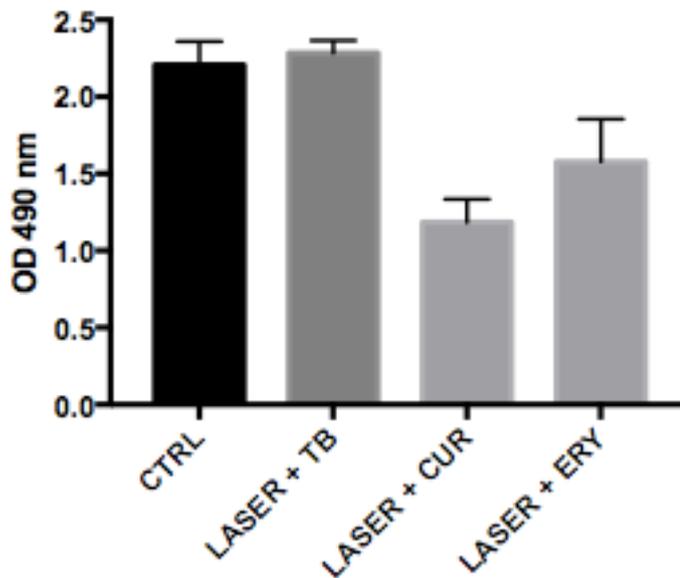


Figure 40. Effects of treatment with laser and dye on *C. albicans* biofilm (Laser + TB, Red diode laser with toluidine blue; Laser + CUR, Blue diode laser with curcumin; Laser + ERY, Green diode laser with erythrosine). ANOVA analysis revealed a statistical significance among the untreated control (CTRL) and groups treated with laser and dye ($p=0.0002$); Tukey's test found a very significant difference between the untreated control group and the group treated with blue diode laser and curcumin ($p=0.0005$) and between the group treated with red diode laser and toluidine blue and the group treated with blue diode laser and curcumin ($p=0.0003$). A statistical significance was found also between the untreated control group and the group treated with green diode laser and erythrosine ($p=0.0114$) and between the groups treated with laser and erythrosine and laser and toluidine blue ($p=0.0058$).

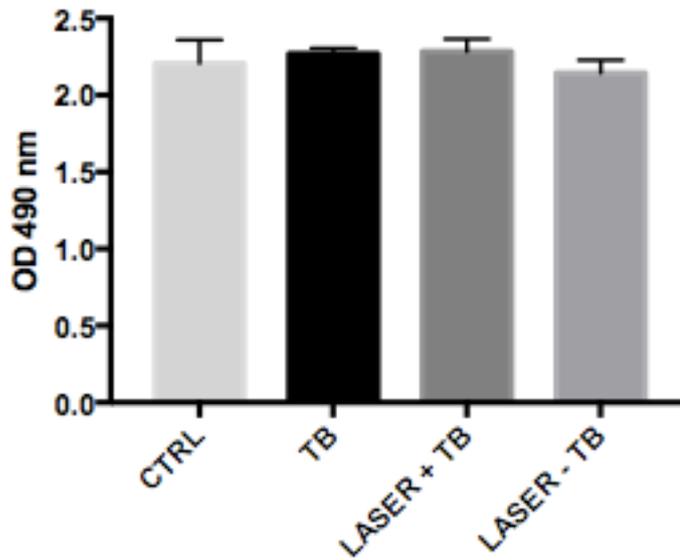


Figure 41. Effects of treatment with toluidine blue alone (TB), combination of red diode laser (650 nm) and toluidine blue (Laser + TB) and red diode laser alone (Laser - TB) on *C. albicans* biofilm. There was no statistical significance among the untreated control (CTRL) and the different treatments.

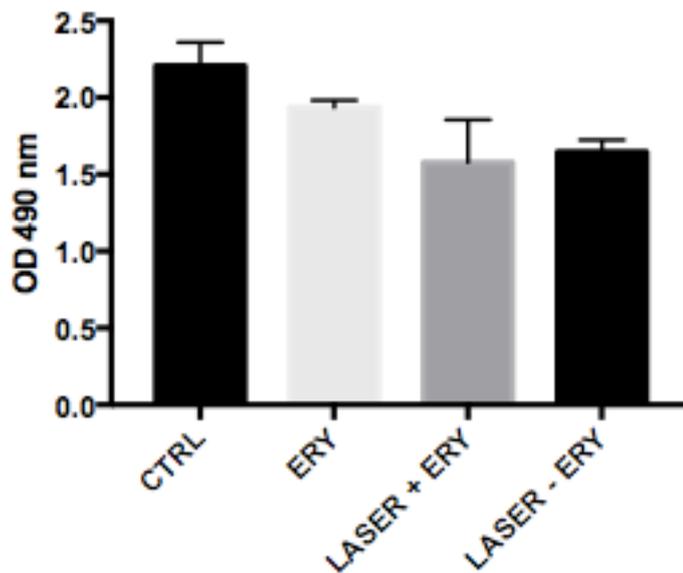


Figure 42. Effects of treatment with erythrosine alone (ERY), combination of green diode laser (532 nm) and erythrosine (Laser + ERY), and green diode laser alone (Laser - ERY) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0055$). Tukey's test found a statistical significance between

untreated control group and the groups treated with green diode laser with erythrosine ($p=0.0068$) and without erythrosine ($p=0.0131$).

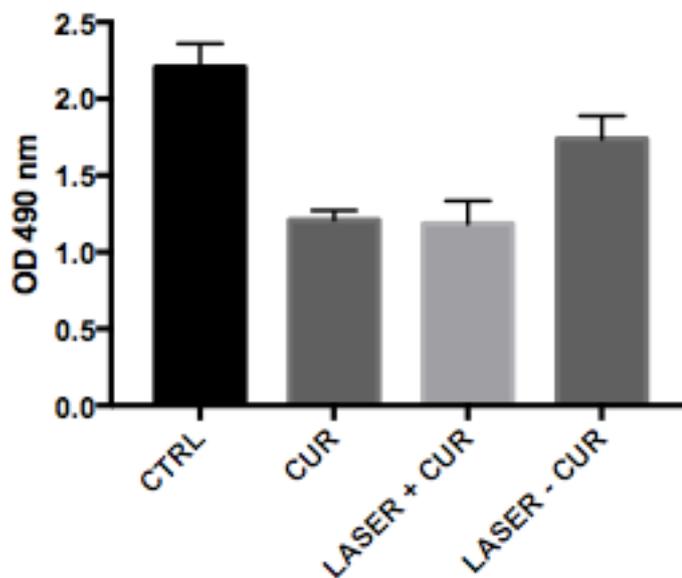


Figure 43. Effects of treatment with curcumin alone (CUR), combination of blue diode laser (405 nm) and curcumin (Laser + CUR), and blue diode laser alone (Laser - CUR) and on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p<0.0001$). Tukey's test found a very significant difference between the untreated control group and the group treated with blue diode laser and curcumin ($p<0.0001$) but also between the untreated control group and the group treated with curcumin alone ($p<0.0001$). A statistical significance was found also between untreated control group and the group treated with blue diode laser alone ($p=0.0114$), between the group treated with curcumin alone and the group treated with blue diode laser without curcumin ($p=0.0057$), and between the groups treated with blue diode laser with and without curcumin ($p=0.0044$).

The results of the treatment with different combinations of red diode laser (650 nm), toluidine blue and KP are shown in Table 7 and Figures 44-52.

In the comparison with the untreated control, the application of red diode laser with or without toluidine blue in combination with KP treatment showed a statistically significant result ($p<0.0001$) (Figure 46), but the combination of dye and KP defined the same significant result without laser application ($p<0.0001$) (Figure 47).

Red diode laser and toluidine blue were more efficient with KP than without KP ($p<0.0001$ vs untreated control) (Figure 48) and red diode laser and KP were efficient in the same mode with or without toluidine blue ($p=0.9758$) with a statistical difference compared to the untreated control ($p<0.0001$) (Figure 49).

Treatment with toluidine blue with KP with or without red diode laser application had an effect on biofilm compared to the control group ($p<0.0001$) (Figure 50) as well as treatment with toluidine blue with or without laser but without KP ($p=0.0136$) (Figure 51) and treatment with KP with or without laser but without toluidine blue ($p<0.0001$) (Figure 52).

Table 7. Effects on *C. albicans* biofilm of treatment with different combinations of red diode laser (650 nm), toluidine blue and KP.

Treatment	OD mean	SD	% Inhibition
Control (no treatment)	3.65	0.049	-
Laser+ TB+ KP-	3.47	0.076	4.94
Laser+ TB- KP-	3.61	0.054	1.1
Laser+ TB- KP+	3.05	0.046	16.44
Laser+ TB+ KP+	3.11	0.082	14.8
Laser- TB+ KP-	3.45	0.057	5.48
Laser- TB+ KP+	2.92	0.109	20
Laser- TB- KP+	2.97	0.114	18.64

Results of the XTT assay are expressed as mean values and standard deviation (SD) of the optical density (OD) at 490 nm, and percentage of inhibition (TB, toluidine blue; KP, synthetic killer decapeptide).

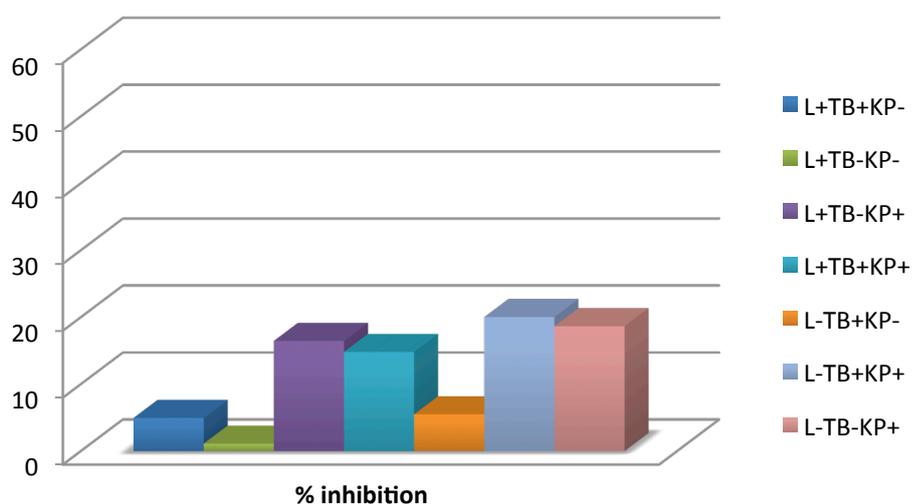


Figure 44. Percentage of inhibition of *C. albicans* SC5314 biofilm after treatment with different combinations of red diode laser (650 nm, L), toluidine blue (TB) and synthetic killer decapeptide (KP) treatment.

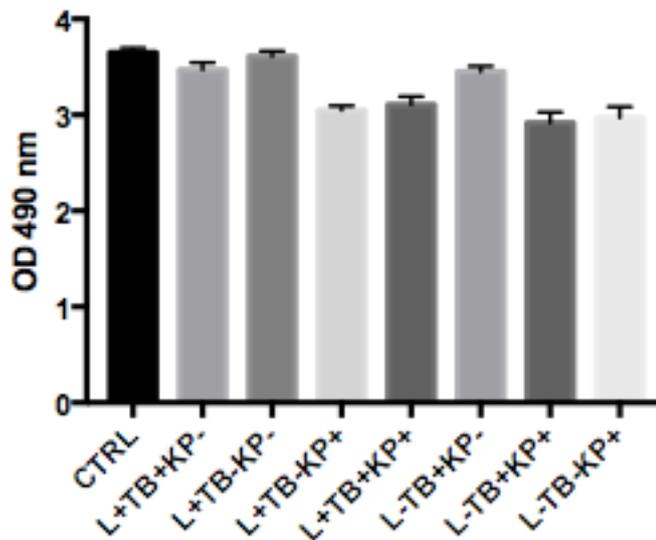


Figure 45. Effects of treatment with different combinations (+ with, - without) of red diode laser (650 nm, L), toluidine blue (TB) and synthetic killer decapeptide (KP) on *C. albicans* biofilm viability. There was a statistical significance among the untreated control (CTRL) and the treatments including KP ($p < 0.0001$).

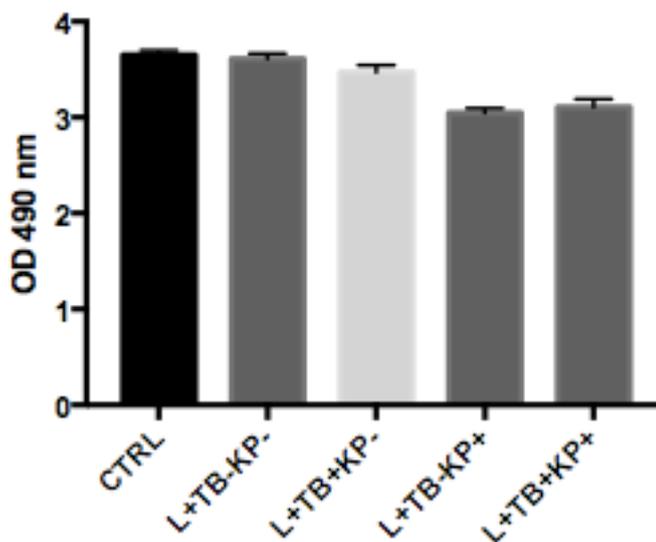


Figure 46. Effects of treatment with red diode laser (650 nm, L), with (+) or without (-) toluidine blue (TB) and synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the treatments including KP ($p < 0.0001$).

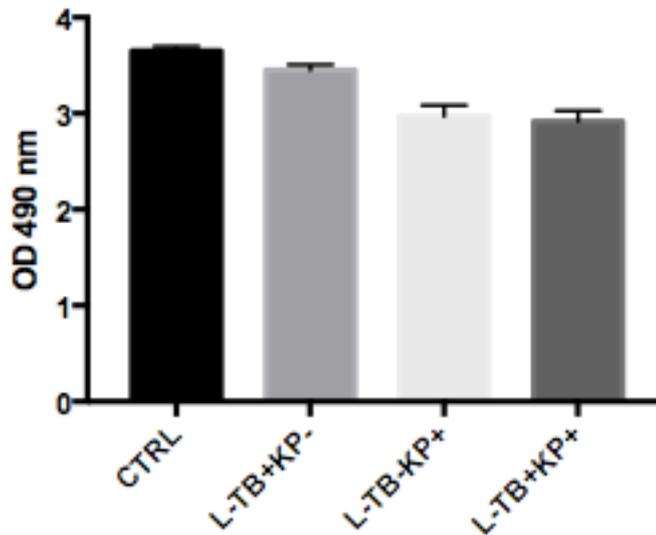


Figure 47. Effects of treatment with different combinations (with +, or without -) of toluidine blue (TB) and synthetic killer decapeptide (KP) without application of red diode laser (650 nm, L) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the treatments including KP ($p < 0.0001$).

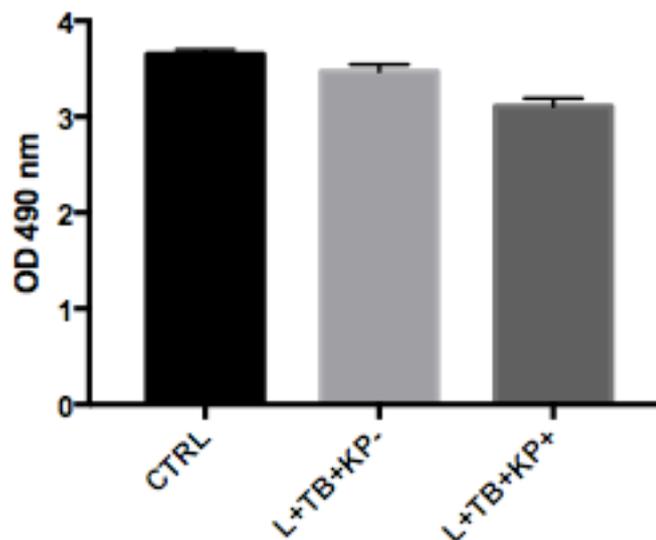


Figure 48. Effects of treatment with red diode laser (650 nm, L) and toluidine blue (TB) with (+) or without (-) synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the treatment including KP ($p < 0.0001$).

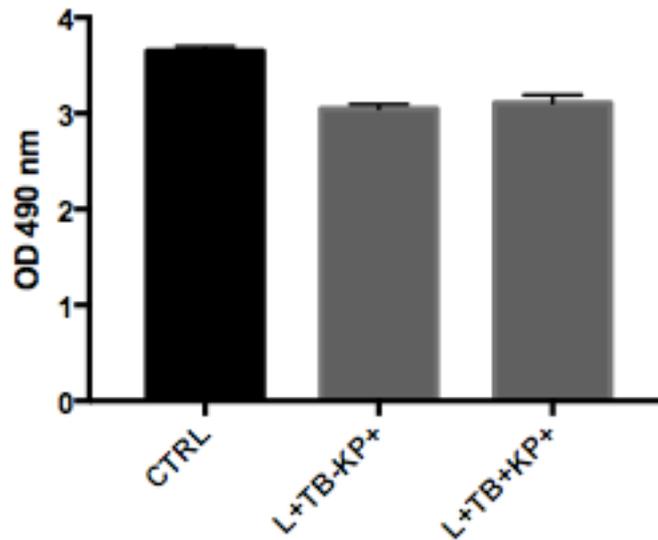


Figure 49. Effects of treatment with synthetic killer decapeptide (KP) and application of red diode laser (650 nm, L) with (+) or without (-) toluidine blue (TB) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p < 0.0001$) but not between the treated groups with or without toluidine blue ($p = 0.9758$).

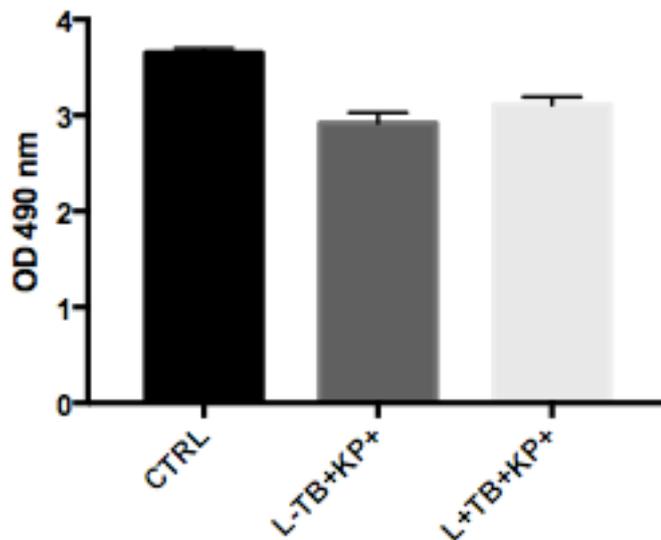


Figure 50. Effects of treatment with synthetic killer decapeptide (KP) and toluidine blue (TB) with (+) or without (-) application of red diode laser (650 nm, L) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p < 0.0001$), but not between the treated groups with or without red diode laser 650 nm ($p = 0.1152$).

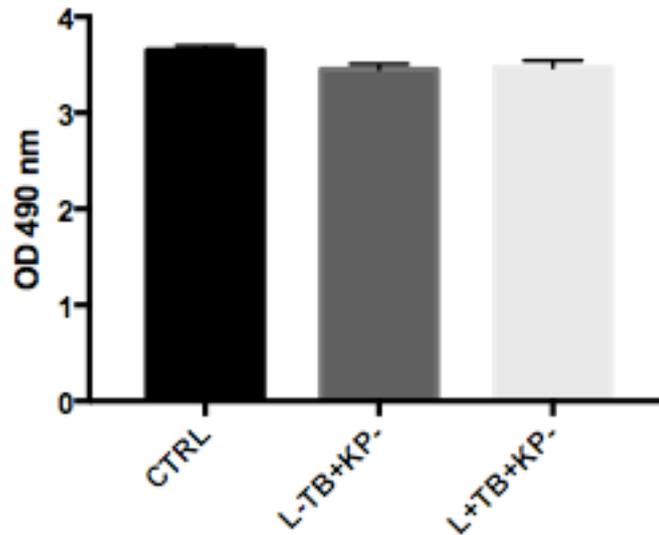


Figure 51. Effects of treatment with toluidine blue (TB) with (+) or without (-) application of red diode laser (650 nm, L) and without synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0136$).

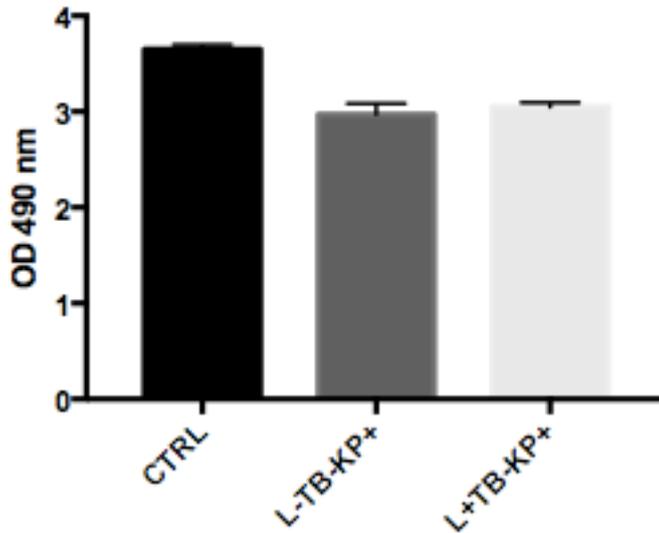


Figure 52. Effects of treatment with synthetic killer decapeptide (KP) with (+) or without (-) application of red diode laser (650 nm, L) without toluidine blue (TB) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p<0.0001$).

The results of the treatment with different combinations of blue diode laser (405 nm), curcumin and KP are shown in Table 8 and Figures 53-61.

In the comparison with the untreated control, the application of blue diode laser with curcumin in combination with KP treatment showed a statistically significant result ($p=0.0006$) (Figure 55), but the combination of dye and KP defined the same significant result without laser application ($p=0.0001$) (Figure 56).

Blue diode laser and curcumin were more efficient with KP than without KP ($p<0.0001$ vs untreated control) (Figure 57) and blue diode laser and KP were more efficient with curcumin than without curcumin with a statistical difference compared to the untreated control ($p=0.0065$) (Figure 58).

Treatment with curcumin with KP with or without blue diode laser application had an effect on biofilm compared to the control group ($p=0.0001$) (Figure 59) as well as treatment with curcumin with or without laser but without KP ($p=0.0001$) (Figure 60) and treatment with KP with or without laser but without curcumin ($p=0.0336$) (Figure 61).

Table 8. Effects on *C. albicans* biofilm of treatment with different combinations of blue diode laser (405 nm), curcumin and KP.

Treatment	OD mean	SD	% Inhibition
Control (no treatment)	3.52	0.008	-
Laser+ CUR+ KP-	2.77	0.072	21.31
Laser+ CUR- KP-	3.37	0.156	4.27
Laser+ CUR- KP+	2.64	0.455	25
Laser+ CUR+ KP+	2.49	0.074	29.27
Laser- CUR+ KP-	2.9	0.093	17.62
Laser- CUR+ KP+	2.55	0.184	27.56
Laser- CUR- KP+	2.92	0.286	17.05

Results of the XTT assay are expressed as mean values and standard deviation (SD) of the optical density (OD) at 490 nm, and percentage of inhibition (CUR, curcumin; KP, synthetic killer decapeptide).

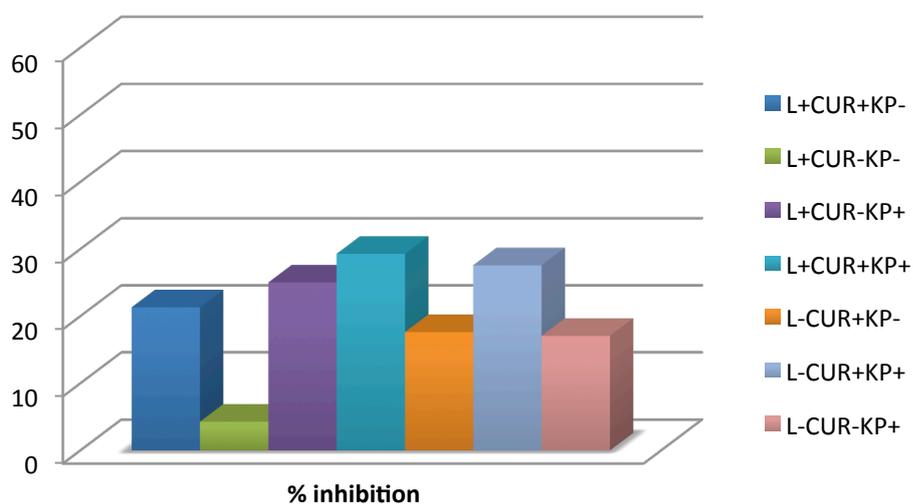


Figure 53. Percentage of inhibition of *C. albicans* SC5314 biofilm after treatment with different combinations of blue diode laser (405 nm, L), curcumin (CUR) and synthetic killer decapeptide (KP) treatment.

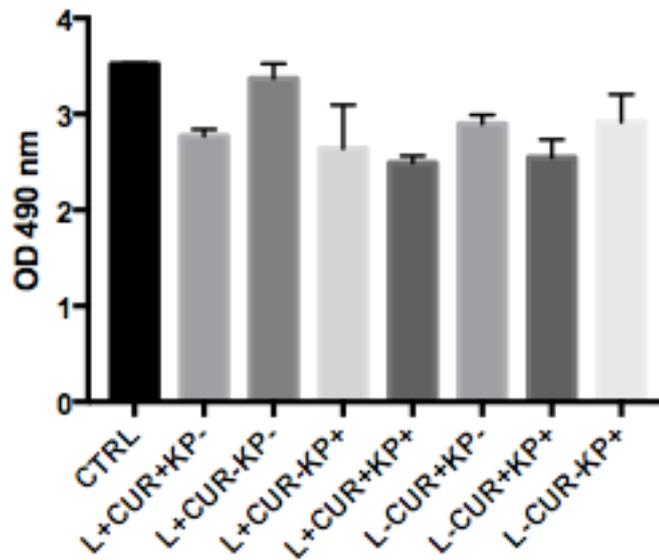


Figure 54. Effects of treatment with different combinations (+ with, - without) of blue diode laser (405 nm, L), curcumin (CUR) and synthetic killer decapeptide (KP) on *C. albicans* biofilm viability. There was a statistical significance among the untreated control (CTRL) and the treatments including KP ($p=0.0001$). Tukey's test revealed a statistical significance in the comparison of the untreated control group and the groups treated with blue diode laser with KP with curcumin ($p=0.0005$) or without curcumin ($p=0.0024$) and in the comparison of the untreated control group and the group treated with curcumin and KP but without laser ($p=0.0009$).

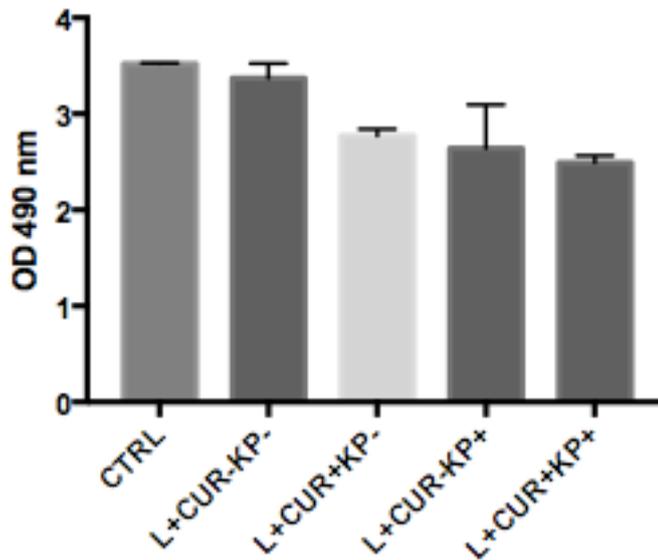


Figure 55. Effects of treatment with blue diode laser (405 nm, L), with (+) or without (-) curcumin (CUR) and synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the other treatments ($p=0.0006$) except for the comparison between the untreated control group and the group treated with laser alone. Tukey's test revealed a statistical significance between the group treated with blue diode laser alone and all the other treated groups.

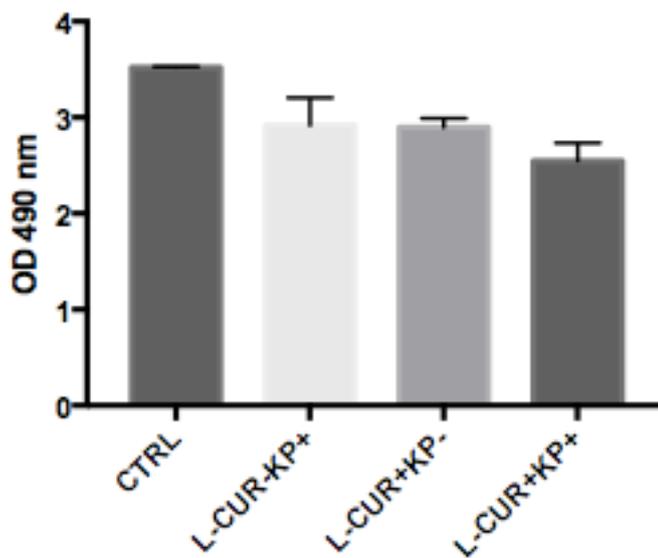


Figure 56. Effects of treatment with different combinations (+ with, - without) of curcumin (CUR) and synthetic killer decapeptide (KP) without application of blue diode laser (405 nm, L) on *C. albicans* biofilm. There was a statistical significance among the untreated control

(CTRL) and the different treatments ($p=0.0001$). Tukey's test found the most significant difference between untreated control group and the group treated with curcumin and KP without laser ($p=0.0007$), but a significant difference was also found between the untreated control group and the groups treated without laser with curcumin and without KP ($p=0.0111$) or without curcumin and with KP ($p=0.0133$).

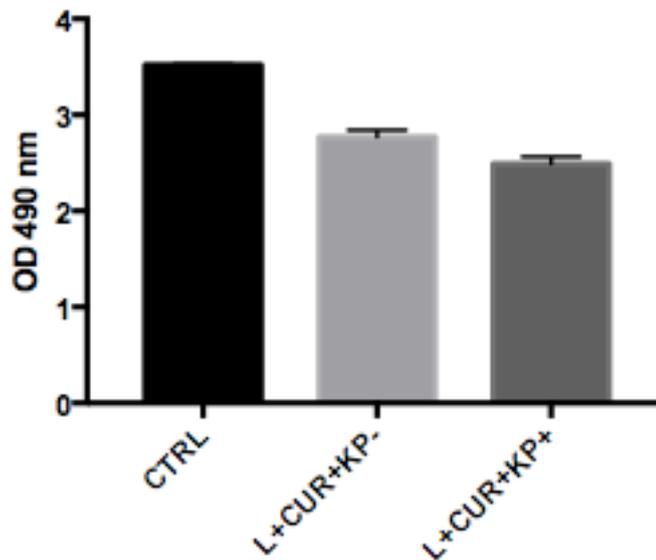


Figure 57. Effects of treatment with blue diode laser (405 nm, L) and curcumin (CUR) with (+) or without (-) synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p < 0.0001$) with a greater effect in the presence of KP ($p=0.0029$) in the comparison between treated groups.

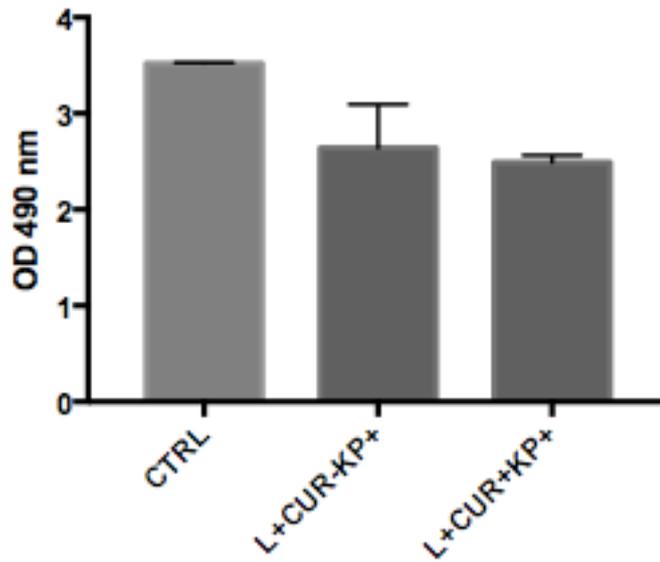


Figure 58. Effects of treatment with synthetic killer decapeptide (KP) and application of blue diode laser (405 nm, L) with (+) or without (-) curcumin (CUR) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0065$), without difference between the treated groups ($p=0.7777$).

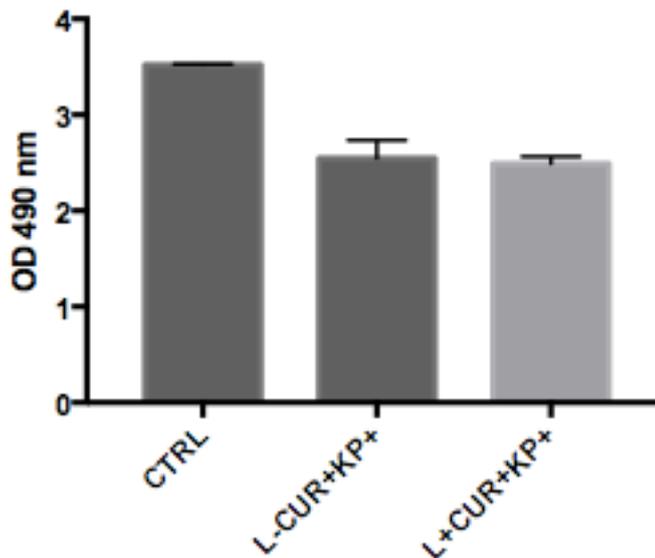


Figure 59. Effects of treatment with synthetic killer decapeptide (KP) and curcumin (CUR) with (+) or without (-) application of blue diode laser (405 nm, L) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0001$) without difference between the treated groups ($p=0.8039$).

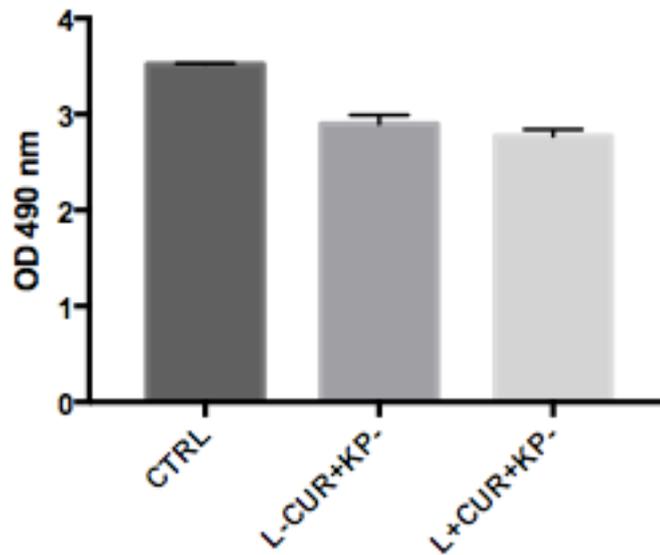


Figure 60. Effects of treatment with curcumin (CUR) with (+) or without (-) application of blue diode laser (405 nm, L) and without synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0001$) without difference between the treated groups ($p=0.1253$).

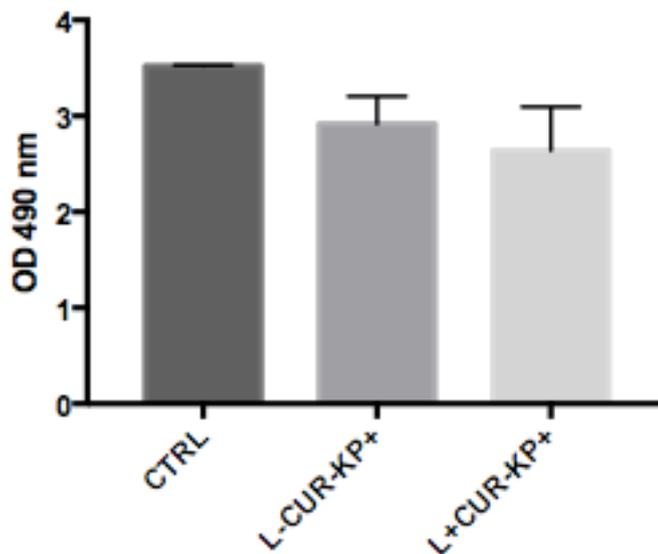


Figure 61. Effects of treatment with synthetic killer decapeptide (KP) with (+) or without (-) application of blue diode laser (405 nm, L) without curcumin (CUR) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different

treatments ($p=0.0336$), with a statistical significance in particular for the comparison between the untreated control group and the group treated with blue diode laser and KP without curcumin ($p=0.0307$).

The results of the treatment with different combinations of green diode laser (532 nm), erythrosine and KP are shown in Table 9 and Figures 62-70.

In the comparison with the untreated control, only the application of green diode laser without erythrosine in combination with KP treatment showed a statistically significant result ($p=0.0001$) (Figure 63).

Treatment with KP did not increase the efficacy of green diode laser and erythrosine compared to control group ($p=0.0889$) (Figure 63). Application of green diode laser in combination with KP treatment was more efficient without erythrosine than with erythrosine ($p=0.0002$) (Figure 64).

Treatment with erythrosine and KP with or without application of green diode laser had no effect on biofilm in comparison to the untreated control ($p=0.062$) (Figure 68) as well as treatment with erythrosine with or without laser application without KP ($p=0.1681$) (Figure 69). KP with or without laser without erythrosine showed an efficacy in comparison to the untreated control ($p=0.0044$) (Figure 70).

Table 9. Effects on *Candida albicans* biofilm of treatment with different combinations of green diode laser (490 nm), erythrosine and KP.

Treatment	OD mean	SD	% Inhibition
Control (no treatment)	3.65	0.039	-
Laser+ ERY+ KP-	3.56	0.044	2.5
Laser+ ERY- KP-	3.57	0.064	2.2
Laser+ ERY- KP+	2.7	0.31	26.03
Laser+ ERY+ KP+	3.47	0.127	4.94
Laser- ERY+ KP-	3.23	0.421	11.51
Laser- ERY+ KP+	3.37	0.148	7.68
Laser- ERY- KP+	3.06	0.194	16.17

Results of the XTT assay are expressed as mean values and standard deviation (SD) of the optical density (OD) at 490 nm, and percentage of inhibition (ERY, erythrosine; KP, synthetic killer decapeptide).

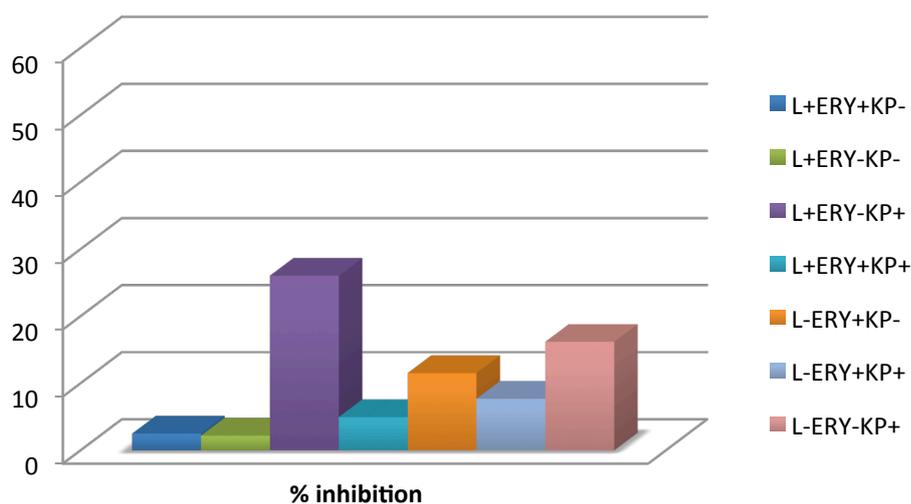


Figure 62. Percentage of inhibition of *C. albicans* SC5314 biofilm after treatment with different combinations of green diode laser (532 nm, L), erythrosine (ERY) and synthetic killer decapeptide (KP) treatment.

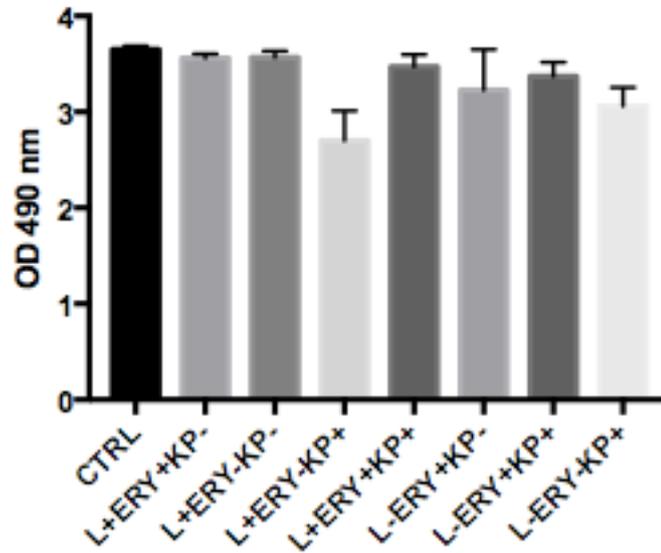


Figure 63. Effects of treatment with different combinations (+ with, - without) of green diode laser (532 nm, L), erythrosine (ERY) and synthetic killer decapeptide (KP) on *C. albicans* biofilm viability. There was a statistical significance among the untreated control (CTRL) and the treatments including KP without erythrosine (ERY) ($p=0.0001$).

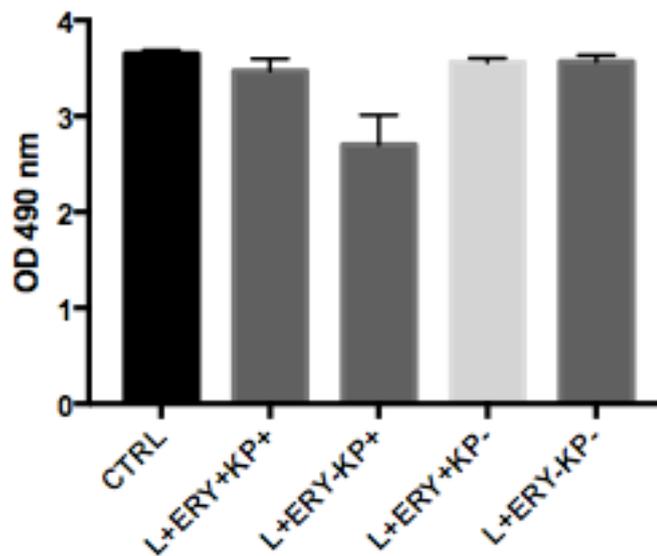


Figure 64. Effects of treatment with green diode laser (532 nm, L), with (+) or without (-) erythrosine (ERY) and synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the treatment including KP

without erythrosine (ERY) ($p=0.0002$). Tukey's test revealed a statistically significant difference between the group treated with green diode laser and KP without erythrosine and all the other treated groups ($p=0.0009$ vs L+ERY+KP+, $p=0.0004$ vs L+ERY+KP-, $p=0.0003$ vs L+ERY-KP-).

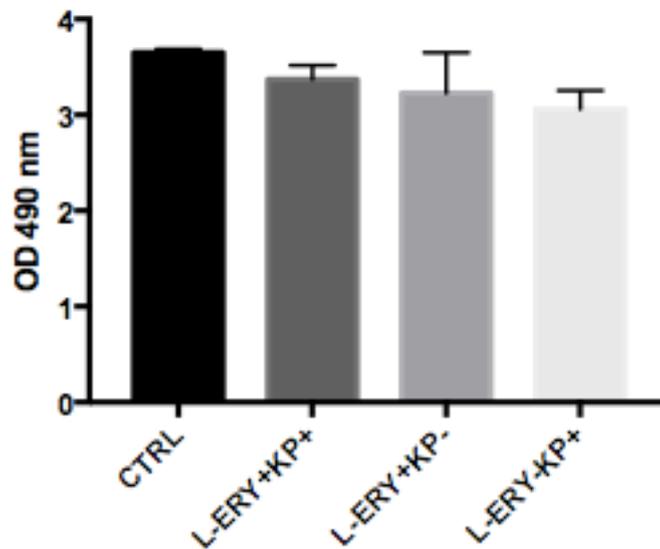


Figure 65. Effects of treatment with different combinations (+ with, - without) of erythrosine (ERY) and synthetic killer decapeptide (KP) without application of green diode laser (532 nm, L) on *C. albicans* biofilm. There was no statistical significance among the untreated control (CTRL) and the treatments.

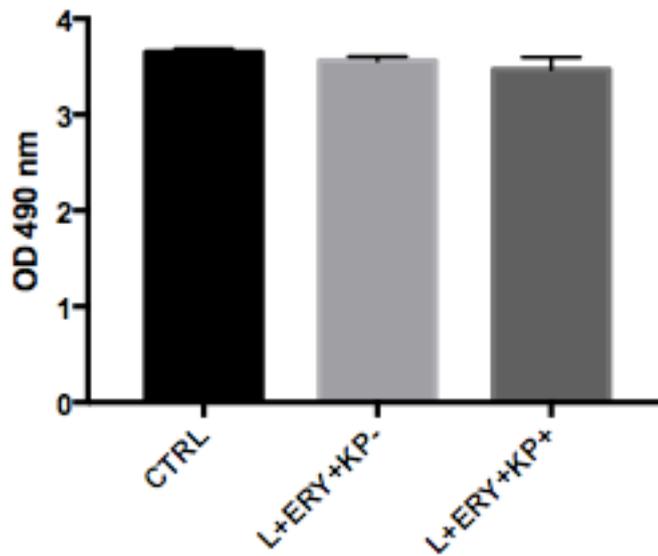


Figure 66. Effects of treatment with green diode laser (532 nm, L) and erythrosine (ERY) with (+) or without (-) synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was no statistical significance among the untreated control (CTRL) and the treatments.

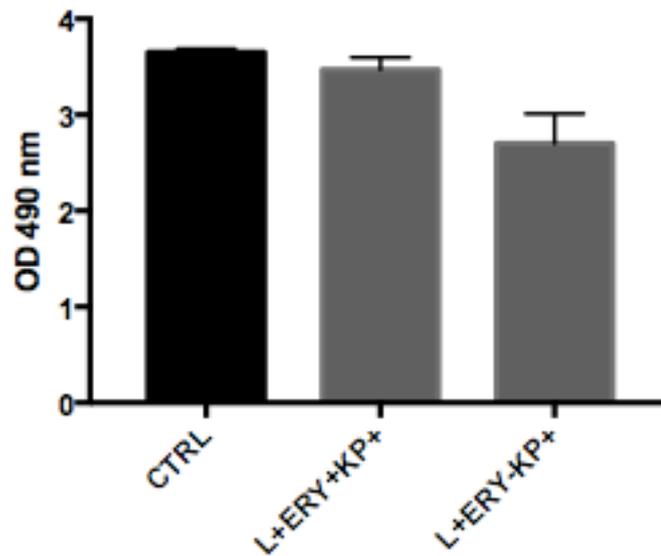


Figure 67. Effects of treatment with KP and application of green diode laser (532 nm, L) with (+) or without (-) erythrosine (ERY) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the treatment without erythrosine ($p=0.0024$), but also between the treatments with and without erythrosine ($p=0.0069$).

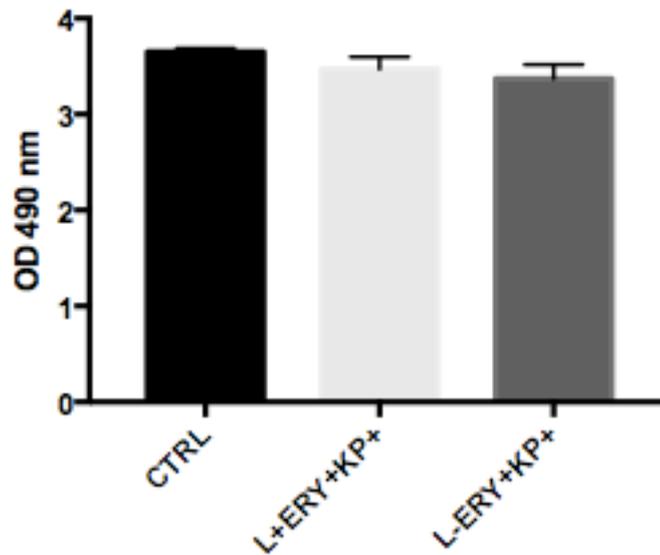


Figure 68. Effects of treatment with synthetic killer decapeptide (KP) and erythrosine (ERY) with (+) or without (-) application of green diode laser (532 nm, L) on *C. albicans* biofilm. There was no statistical significance among the untreated control (CTRL) and the treatments.

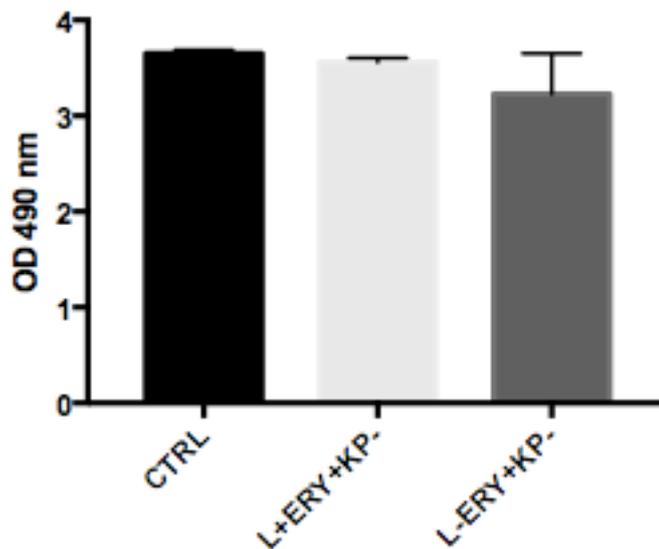


Figure 69. Effects of treatment with erythrosine (ERY) with (+) or without (-) application of green diode laser (532 nm, L) and without synthetic killer decapeptide (KP) on *C. albicans* biofilm. There was no statistical significance among the untreated control (CTRL) and the treatments.

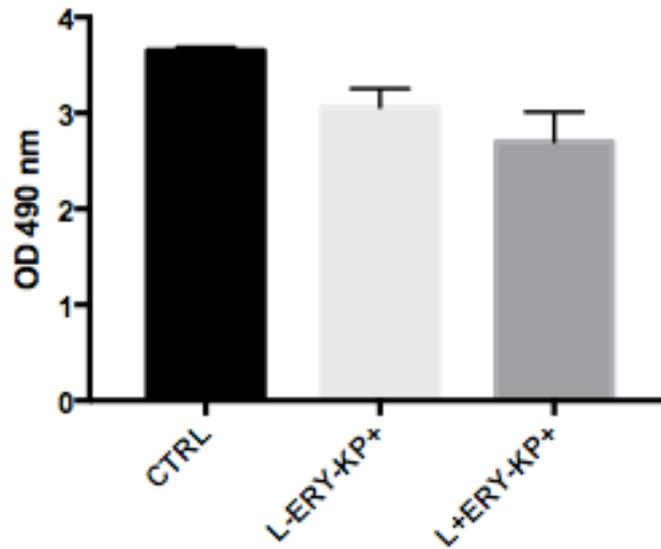


Figure 70. Effects of treatment with synthetic killer decapeptide (KP) with (+) or without (-) application of green diode laser (532 nm, L) without erythrosine (ERY) on *C. albicans* biofilm. There was a statistical significance among the untreated control (CTRL) and the different treatments ($p=0.0044$), particularly versus the group treated with laser and KP without erythrosine ($p=0.0037$).

Discussion

In preliminary studies, three different wavelengths were applied with or without photosensitizers on *C. albicans* cells *in vitro*. No growth inhibition was observed without photosensitizers, thus the first result of the *in vitro* study on planktonic cultures is that laser did not cause a growth inhibition in saline suspension without photosensitizer.

Moreover, for all wavelengths we used, laser alone without photosensitizer stimulated *C. albicans* growth, with an increase of CFU number, particularly with red and green diode. This is a different reaction compared to the report by Souza and co-workers in 2010 who described a decrease of CFU number after use of a 660 nm laser without photosensitizer at 15.8, 26.3 and 39.5 J/cm². One hypothesis for this result may be the minimal thermal increase given by the use of laser without photosensitizer with a biomodulatory effect on *C. albicans* cells. The maximum inhibition of growth of *C. albicans* in saline solution (100%) was achieved with the blue diode at any used fluence and curcumin, a result confirmed by the evaluation of the inhibition areas obtained on solid medium.

Red diode laser (650 nm) used with toluidine blue caused a growth inhibition variable between 79.31% and 95.79%, although no inhibition was observed on solid medium. The candidacidal effect was dose-dependent with an increase of killing from 10 to 30 J/cm², as already reported in literature also for other wavelength-photosensitizer combination on bacterial cultures (Hamblin MR and

Hasan T 2004, Gad F et al 2004).

It is to be emphasized that the coupling of red diode laser at 10 J/cm² and toluidine blue did not improve the effect obtained with toluidine blue alone, while better results were obtained at 20 and 30 J/cm². Our results, with an inhibition value of 95.79% for 650 nm diode confirm other results obtained with similar wavelengths in red spectrum as He-Ne laser (Wilson M and Mia N 1993) or with similar photosensitizers as methylene blue (Souza RC et al 2010).

Conversely, green diode laser used with erythrosine led to a growth inhibition variable between 10.34% and 39.85% in saline solution, while very clear and large inhibition areas were observed on solid medium. In this condition the coupling of green diode and erythrosine gave the best result at 30 J/cm², while erythrosine alone had no candidacidal effect.

The explanation for these apparently contradictory results may be related to some differential reaction of the dyes in saline suspension and in solid medium: this may be the subject for future studies finalized to clarify the mechanism of photodynamic inactivation against *C. albicans*.

Blue diode laser (405 nm) showed a complete candidacidal effect already at the lower fluence.

Martin CV and coworkers in 2009 reported that curcumin is 2.5 fold more potent than fluconazole at inhibiting the adhesion of *C. albicans* to buccal epithelial cells. Curcumin acts as antifungal by generating reactive oxygen

species, triggers an early apoptosis in *C. albicans* cells and affects membrane associated ATPase activity, ergosterol biosynthesis and protein secretion: this properties seem to be improved by the action of laser light, explaining the candidacidal effect found in our study.

The experimental *in vivo* study exploited the recently adopted non-vertebrate *G. mellonella* model to explore the efficacy of laser PDT against *C. albicans* infection.

The *G. mellonella* model provides many competitive advantages over mammalian models, such as an important reduction in time and cost and lack of ethical or legal concerns, other than simplicity to treat (e.g. without anesthesia) and maintain a large number of animals.

Preliminary studies demonstrated that no one of the used dyes was toxic for *G. mellonella*, while in infected larvae a difference in survival was obtained only for the group treated with red diode laser and toluidine blue in comparison to toluidine blue and laser used alone. The absence of statistically significant difference between the survival of untreated infected group and the dye and/or laser treated infected groups was probably due to the uncommon survival curve obtained for *G. mellonella* larvae infected with *C. albicans* and not treated, compared with that of literature and previous studies.

In a second phase of the study, we observed for every treatment applied (laser alone, dye alone or combination of dye and laser) a significant difference in

survival curves in comparison to the control untreated group.

Infected larvae inoculated with erythrosine alone showed a prolonged survival curve in comparison to those inoculated with erythrosine and treated with 532 nm diode laser, although a statistically significant difference ($p=0.03$) in survival was found only between the group of larvae irradiated with 532 nm diode laser in respect to the group treated with laser coupled to erythrosine. One hypothesis for this result may be that the combination of laser and erythrosine could create some alterations in the dye molecule decreasing the candidacidal effect.

A similar hypothesis could be formulated for blue laser and curcumin, where laser application alone resulted in a prolonged survival, but a statistically significant difference ($p=0.02$) was found only between the group inoculated with curcumin alone in comparison to the group treated with 405 nm diode laser coupled with curcumin. These results are comparable with those of Carmello et al who reported better results for blue light alone rather than in combination with curcumin, maybe for the complex mechanisms and interactions between photosensitive compound and laser (Carmello JC et al 2015).

Instead, the combination of toluidine blue and 650 nm diode laser application led to a prolonged survival with respect to dye alone or laser application alone, although the difference in survival was not statistically significant.

The insect immune response demonstrates structural and functional similarities

to the innate immune response of mammals and, in particular, insect haemocytes and mammalian neutrophils have been shown to phagocytize and kill pathogens in a similar manner. Recent studies demonstrated that PDT can stimulate host defense mechanisms via the attraction and accumulation of neutrophils into the infected region: this may explain the results obtained in *G. mellonella* for the different treatments (with or without laser and photosensitizers) (Junqueira JC 2012, Chibebe J et al 2013).

Other studies will be necessary to validate these results, and to better understand the interaction among animal host, *C. albicans*, photosensitizers and lasers. PDT protocols could be also evaluated *versus* or in combination with antifungals.

With regard to the effects on *C. albicans* biofilms, the combination of 650 nm red laser and toluidine blue, as well as the application of red laser alone and toluidine blue alone, did not show any result in terms of reduction of cell viability in the XTT assay, although SEM experiments led to hypothesize an inhibitory activity on biofilm formation, in particular for combined treatment. Conversely, combination of 405 nm blue diode laser with curcumin and curcumin alone showed interesting results with a reduction of viability of about 46-47%; the worst result for this PDT protocol was obtained for the use of laser alone without PS (viability of about 79%).

Green diode laser in association with erythrosine reduced vitality until 71.5%, but without erythrosine was about 75%.

Following studies confirmed some previous result, as the absence of any effect on *C. albicans* inhibition for the combination of red diode laser application and toluidine blue; the best result obtained with the introduction of KP in the *C. albicans* biofilm model was an inhibition of about 20% with KP and toluidine blue, a result similar to that obtained using only KP.

The introduction of KP in biofilm model in the protocol coupling green diode laser and erythrosine gained the best results with laser with KP and without erythrosine with an inhibition of about 26%, with a result comparable to the one obtained in biofilm model without introduction of KP.

As in previous studies, also in the studies with the introduction of KP in the *C. albicans* biofilm model, the combination of 405 nm blue diode laser and curcumin obtained best result with an inhibition of 30% in presence of curcumin, laser and KP.

Some studies in the past suggested that curcumin alone, also at low concentrations (100 μ M), may have antifungal properties that are improved and increased in combination with light (Martins CV et al 2011): in the study we conducted on planktonic cultures curcumin alone at the concentration we used it (100 μ M) did not inhibit *C. albicans*. Moreover, we obtained with parameter lower than that reported in the literature (Martins CV et al 2009) maximal results in terms of percentage of inhibition (100%).

About results concerning toluidine blue, for planktonic cultures we obtained

results on inhibition until about 83% in planktonic cultures, but without any positive results neither in planktonic (17% of inhibition) or biofilms (<20% of inhibition in combination with red laser and KP) cultures. This is in contradiction with the study of Pupo YM et al of 2011 where authors reported a number of viable *C. albicans* cells significantly reduced after PDT in a mode regardless of the photosensitizer used.

Previous studies reported that microorganisms organized in biofilms are also less susceptible to photodynamic procedure compared with planktonic phase because of the heterogeneity of the biofilm, the reduced growth rate of cells, the differences in gene expression, and the limited penetration of antimicrobial agents across the extracellular matrix material (Rossoni RD et al 2014). Also in our experience there is a gap in terms of inhibition of *C. albicans* in planktonic culture or in biofilms: for example, where application of blue diode laser and curcumin gave 100% of inhibition on planktonic cultures, the same combination of laser and dye with the same parameter on biofilm gave an inhibition of about 30% with the addition of KP.

Literature describes different times of preirradiation, affirming that killing is not preirradiation time-dependent for planktonic cultures but it must be higher in biofilms cultures (Silva MP et al 2016): this may be a key point to improve results in the biofilm model.

APDT is reported in literature as effective against bacteria resistant to antibiotics

and not inducing the development of resistant strains (Wainwright M and Crossley KB 2004, Hamblin MR and Hasan T 2004, O'Riordan K et al 2005, Silverman S 1999, Komerik N and MacRobert AJ 2006). In fact, the resistance to this treatment is unlikely, given that the singlet oxygen and free radicals interact with different cell structures and different metabolic pathways (Hamblin MR et al 2004, O'Riordan K et al 2005, Silverman S 1999, Komerik N and MacRobert AJ 2006).

The presence of several and different cellular targets in fungi may reduce the risk of selection of photomutant resistant strains with a further reduction for this risk thanks to the lack of mutagenic effect for PDT (Calzavara-Pinton PG et al 2005).

It is reported that photosensitizer and low laser doses described as efficient in killing yeast *in vitro* do not kill human cells also because the absence of genotoxic or mutagenic effect (Donnelly RF et al 2008).

Moreover, PDT is low cost and possesses low overdose risk.

APDT may be a good alternative to antimicrobial drugs, given the possible acquired resistance, especially for the treatment of localized infections of the skin and oral cavity.

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