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Algicidal effect of blue light on pathogenic *Prototheca* species

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ABSTRACT

Prototheca spp. are pathogenic algae with important zoonotic potential. Most importantly, these algae often infect dairy cattle. Since there is no effective therapy against the algae, the standard recommendation is the disposal or culling of infected cows to avoid outbreaks. This study investigated the ability of blue light to inactivate pathogenic *Prototheca* species. Blue LED light ($\lambda = 410$ nm) was used to inactivate *in vitro* suspensions of *P. zopfii* genotypes 1 and 2, and *P. blaschkeae*. Our results showed that blue light irradiation induced a strain-specific dose-dependent algicidal effect against all tested strains. *P. zopfii* genotype 1, was more sensitive than genotype 2 and *P. blaschkeae* was the most tolerant. Even though we observed different inactivation kinetics, all strains presented significant photoinactivation levels within feasible procedure periods. Therefore, we conclude that blue light irradiation offers promising potential for the development of novel technologies that control contaminations and infections caused by *Prototheca* spp.

1. Introduction

Prototheca spp. are unicellular, achlorophyllous, yeast-like microalgae that occur ubiquitously in nature, on all continents except Antarctica [1]. They are saprophytes, occupying mostly aqueous environments with high organic matter content [2]. *Prototheca* spp. are opportunistic pathogens that cause infections in humans and domestic animals [1–3].

Human protothecosis is manifested as cutaneous, articular, and disseminated disease, although it remains a rare clinical condition. Human infections occur through direct contact with contaminated sources or upon traumatic inoculation with the pathogen. However, the pathogenic mechanisms of the protothecal diseases remains unclear [1,2].

To date, eight species have been recognized: *P. stagnora*, *P. ulmea*, *P. tumulicola, P. cutis*, *P. miyajii*, *P. wickerhamii*, *P. zopfii*, and *P. blaschkeae* [1,4,5], and one subspecies *P. blaschkeae* subsp. *brasiliensis* [6]. Of these, *P. wickerhamii*, *P. cutis*, and *P. miyajii* have been mainly related to human infections [1,7], whereas *P. zopfii* and *P. blaschkeae* [4,8,9] with its subspecies *P. blaschkeae* subsp. *brasiliensis* have been responsible for bovine mastitis [6].

Although bovine mastitis is the predominant form of protothecal infections in animals, the disease has also been described in dogs, cats, and goats. Single cases of *Prototheca* infections have been reported in a deer, a beaver, a fruit bat, a snake, and more recently in a fish and horses [2,10,11].

In dairy cows, protothecosis has become an emerging problem as the prevalence of mammary infections increases globally [1,9,6,7]. Disturbingly, bovine protothecosis often resists most of the commercially available antimicrobials [9,12–14,6]. The chronic infections and resistance of *Prototheca* species to conventional drugs is attributed to the establishment of pyogranulomatous inflammation [15] and the evasion of the algae from phagocytic cells [16]. In addition, biofilm production can allow their persistence in the cattle environment making control and eradication of the disease on dairy farms extremely difficult [17,18].

This scenario has motivated a number of studies exploring *in vitro* effects of antimicrobials, antiseptics, disinfectants, and other alternative approaches against pathogenic *Prototheca* spp. [7,9,19–22]. In this regard, our group has already demonstrated the algicidal effect of antimicrobial photodynamic therapy (aPDT) mediated by exogenous photosensitizers (PSs) against *Prototheca* strains [20]. However, the use

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of PSs is a limiting factor to introduce aPDT into dairy industry since they represent residues that significantly affect organoleptic properties of dairy products [23].

aPDT mediated by blue light irradiation and PSs derived from porphyrins have been broadly investigated over the last decades. In addition, blue light alone has long been recognized to have intrinsic antimicrobial properties in different microorganisms, including bacteria, viruses, and fungi ([24–26]). The mechanism of action still remains unclear, although the most accepted hypothesis is that the blue light excites naturally occurring pigments (porphyrins and/or flavins) that are abundant in microbial cells. This interaction leads to the production of reactive oxygen species (ROS) that results in cellular damage of multiple structures (*e.g.* proteins, DNA and lipids), and subsequent cell death [24]. In this study, we investigated for the first time the algicidal effect of blue light on pathogenic *Prototheca* species isolated from dairy cows.

2. Materials and methods

We used three *Prototheca* type strains: *P. zopfii* genotype 1 (RZI-3), *P. zopfii* genotype 2 (LZ-5), and *P. blaschkeae* (RZIII-3). Fresh colonies of each strain were picked from Saboraud Dextrose agar (Difco Laboratories, Detroit, USA), inoculated into 10 mL of Sabouraud Dextrose broth and incubated at 37 °C for 48 h under continuous shaking regimen (100 RPM). The cultures were then washed twice in PBS (phosphate buffer solution, pH 7.4) with 0.05% Tween 80. For each washing step, the cells were collected by centrifugation at 10.000 RPM for 10 min. After the second washing step, cells were suspended in PBS with 0.05% Tween 80 to prepare the experimental inoculum. The inocula were standardized to 10^5 colony forming units per milliliter (CFU/mL) using a spectrophotometry calibrated at 540 nm to adjust to an optical density of 0.08.

One -mL of each sample was individually placed in 24-well plates for blue light irradiation procedure. A homogeneous irradiation system of high-power LEDs was used with emission wavelength of 410 +/- 10 nm and irradiance of 38.2 mW/cm^2 (LEDbox, BioLambda, São Paulo, Brazil). Experimental groups with 20-minute intervals were compared to the respective control groups (C) that were kept in the dark for the entire experimental procedure (Table 1).

Post-treatment samples were serially (1:10) diluted in PBS and inoculated onto Sabouraud Dextrose agar plates, incubated at 37° C for 48 h, and the cell count (CFU/mL) was determined as described by Jett et al. [27]. All tests were performed in triplicates performed on three separate days.

Survival fraction values were defined as the $\log_{10}$ reduction of CFU normalized in relation to control groups. Survival fractions were analyzed by Shapiro-Wilk test to confirm normality and compared between strains using two-way analysis of variance (ANOVA) with Tukey's post-hoc test. Statistical tests were performed using Prism software (GraphPad, USA). The level of statistical significance was set at 0.05 for all tests.

3. Results and discussion

Blue light irradiation showed a clear algicidal effect against all mastitis-related *Prototheca* strains. The analysis revealed significant

Table 1
Time and radiant exposure (J/cm^2) of blue light applied for the establishment of death curve of *Prototheca* species.

	Irradiation parameters						
Minutes	20	40	60	80	100	120	140
J/cm^2	45.83	91.67	137.50	183.34	229.17	275.01	320.85

J = Joules.

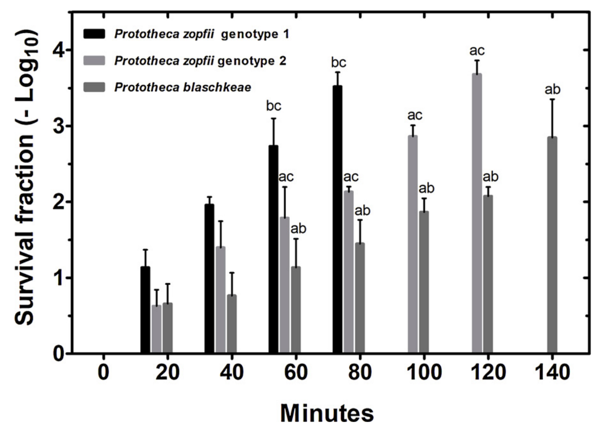

Fig. 1. Survival fraction of *Prototheca* species inactivation induced by blue light (λ = 410 nm) irradiation. The results are presented as survival fractions of $\log_{10}$ reduction in function of irradiation time. The error bars indicate standard deviation of the mean at each data point.

variation in survival fractions within the irradiation doses used. In fact, we could also observe a strain-specific inactivation kinetics.

All experimental groups presented significant differences in relation to their respective control groups. At first 40 min, no significant difference between inactivation rates of different strains was observed (Fig. 1). However, after 60 min of irradiation (137.5 J/cm^2) each strain exhibited a different inactivation rate. *P. zopfii* genotype 1 was the most sensitive, presenting more than 3 logs of inactivation after 80 min. exposure to (183.34 J/cm^2) irradiation. Likewise, *P. zopfii* genotype 2 required 120 min (275 J/cm^2) to reach more than 3 logs of inactivation. *P. blaschkeae* was significantly more tolerant to blue light inactivation. For this species, more than 2 logs of inactivation occurred only after 140 min (320.85 J/cm^2). At the same dose, *P. zopfii* genotypes 1 and 2 did not yield any CFU counts. These particular findings suggest that each species and strain have intrinsic biochemical characteristics that determine their sensitivity to blue light unique. This phenomenon may be related to the content of antioxidant systems and concentration of photosensitive pigments. However, the quantification of such variables should be investigated in future studies.

According to the literature, *P. zopfii* and *P. wickerhamii* have been shown inactivated through aPDT using exogenous prodrugs such as 5-aminolevulinic acid [22] and PS such as methylene blue [20] combined with red light irradiation. In the present report, we demonstrated for the first time the algicidal effects of blue light alone, against pathogenic *Prothoteca* strains.

The inhibitory effect of blue light on microorganisms has been evidenced as early as in the beginning of the last century [28]. In fact, the 1903' Nobel Prize of Medicine was granted to Niels Finsen for the treatment of cutaneous tuberculosis by exposure to blue light. Even though, the biochemical mechanisms of blue light inactivation remain poorly understood. Current literature supports that excitation of endogenous pigments by light leads to photochemical reactions in an analogous manner as photodynamic therapy with exogenous dyes occurs. Hence, the most pronounced effect caused by blue light alone should be linked with the most intense absorption bands of ubiquitous and abundant pigments such as porphyrin and flavin derivatives [24]. On the other hand, blue light presents lower penetration into tissues than red light. Such phenomenon is associated to the interference of tissue proteins that strongly absorb and scatter blue light. This limitation may restrict the use of blue light approach to treat superficial infections and contaminations.

Blue light inactivation has now returned as a trending antimicrobial technique due to the emergence of drug resistance allied to the technological revolution of light emitting diodes (*e.g.* LED and laser). Yang et al. [29] have recently demonstrated that blue light is active against suspensions and biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA). The same study showed promising *in vivo* results of the blue light in murine skin burns infected by MRSA. Interestingly, important environmental contaminants, such as sporulating bacteria, have also been shown susceptible to blue light [30]. Hence, the broad-range microbicidal activity and lack of microbial resistance to blue light has attracted much attention as a potential therapeutic platform for many superficial infections and contaminations.

Prototheca species present intrinsic resistance to most antimicrobial agents currently used in veterinary medicine [1,3], resulting in the disposal or culling of infected cows. This issue includes drugs, antiseptics, and disinfectants [7,9,19]. Moreover, *Prototheca* spp. may resist heat treatments applied for milk and dairy products, posing a potential risk of gastroenteritis to human consumers [8,12].

Since the algae are considered as environmental pathogens, their presence in the dairy farm surrounding increases the risk of dissemination throughout the herd [13]. Furthermore, the ability of *Prototheca* spp. to produce biofilms makes them extremely difficult to eradicate from milking machines and other dairy farm utensils [18]. In this context, we suggest that future studies should also address the effects of blue light against *Prototheca* biofilms as a potential strategy to eliminate the most persistent surface contaminations.

Given the important losses incurred by protothecal mastitis on the dairy industry, along with its rising impact on human health [1,6], blue light seems to be an interesting alternative approach. The optimization of irradiation parameters and the use of more refined and targeted light delivery systems (*e.g.* modified optical fibers, milking and storage machinery equipped with blue light sources) further enhances the potential of blue light as an antimicrobial tool.

In summary, all *Prototheca* strains tested showed significant inactivation rates in a dose-dependent manner. This study is the first to demonstrate an algicidal effect of blue light irradiation against *Prototheca* algae. Therefore, the confirmed antimicrobial potential of blue light opens a new perspective for the development of novel technologies applicable in veterinary medicine and dairy industry.

Conflict of interest

C. P. Sabino is an associate at BioLambda but declares to only have scientific interest on this study. There are no further conflicts of interest to be declared.

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